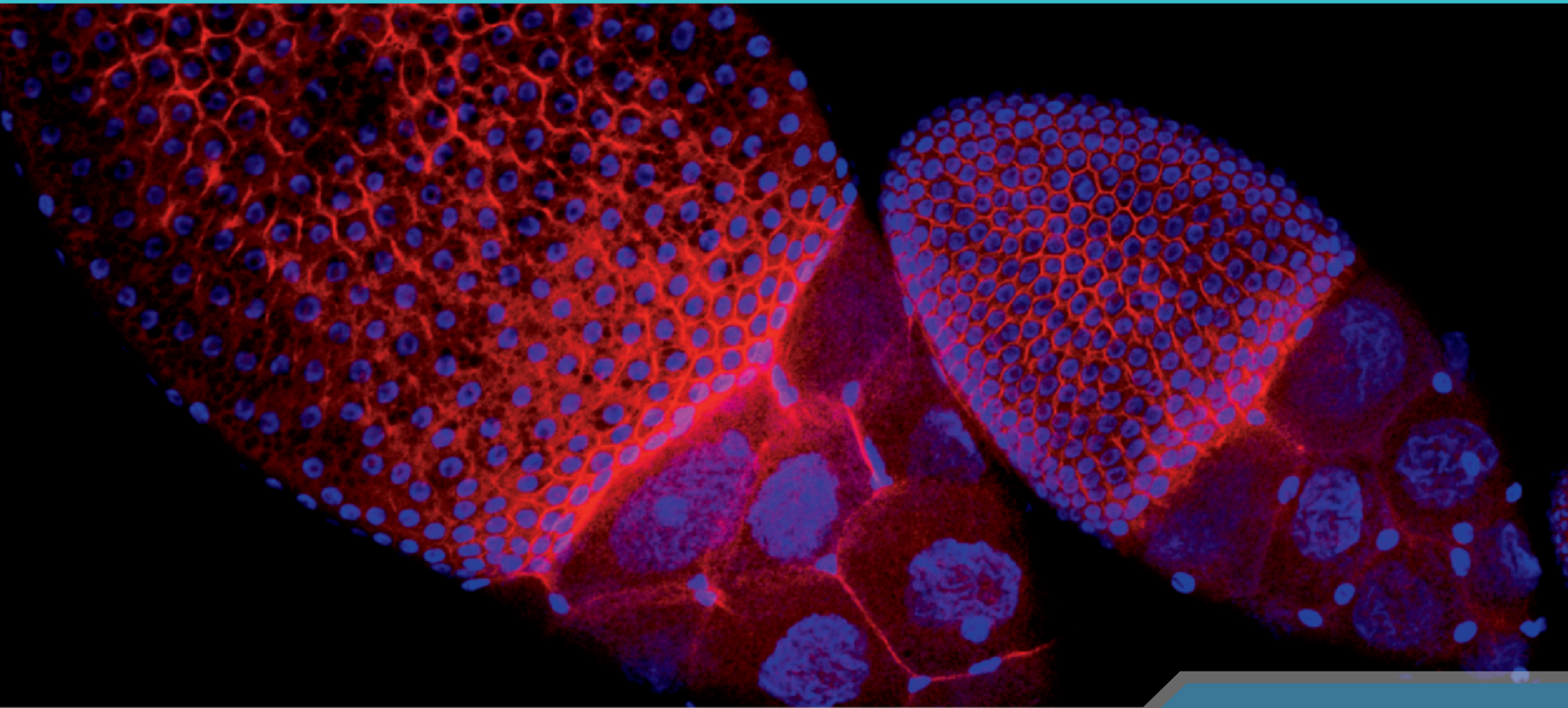


# CST Protocols & Troubleshooting Guides





**Production Team**

Troy Humphreys PhD, Product Scientist (left)  
Christina Rosi, Product Scientist (center)  
Kenneth Gilbert, Production Associate (right)

## CST Validation = Reagents + Protocol •

Cell Signaling Technology approaches validation from the perspective of the scientist, because that is who we are. We believe it takes more than just seeing a band at the expected molecular weight to validate antibody specificity. Our scientists validate all of the products from CST in-house for quality and specificity across broad applications. The result is a set of optimized protocols tested with every product. Using CST validated products with CST validated protocols provides reproducible and reliable results. And just in case an unexpected result arises, the same scientists who validate our reagents and use these protocols every day are available to provide you with technical support.



# Western Immunoblotting (WB)

## Chemiluminescent

last updated: 8/21/13

For western blots, incubate membrane with diluted primary antibody in either 5% w/v BSA or nonfat dry milk, 1X TBS, 0.1% Tween® 20 at 4°C with gentle shaking, overnight. **NOTE:** Please refer to primary antibody datasheet or product webpage for recommended primary antibody dilution buffer and recommended antibody dilution.

### A. Solutions and Reagents

**NOTE:** Prepare solutions with reverse osmosis deionized (RODI) or equivalent grade water.

- 1. 20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L 1X PBS: add 50 ml 20X PBS to 950 ml dH<sub>2</sub>O, mix.
- 2. 10X Tris Buffered Saline (TBS):** (#12498) To prepare 1 L 1X TBS: add 100 ml 10X to 900 ml dH<sub>2</sub>O, mix.
- 3. 1X SDS Sample Buffer:** Blue Loading Pack (#7722) or Red Loading Pack (#7723) Prepare fresh 3X reducing loading buffer by adding 1/10 volume 30X DTT to 1 volume of 3X SDS loading buffer. Dilute to 1X with dH<sub>2</sub>O.
- 4. 10X Tris-Glycine SDS Running Buffer:** (#4050) To prepare 1 L 1X running buffer: add 100 ml 10X running buffer to 900 ml dH<sub>2</sub>O, mix.
- 5. 10X Tris-Glycine Transfer Buffer:** (#12539) To prepare 1 L 1X Transfer Buffer: add 100 ml 10X Transfer Buffer to 200 ml methanol + 700 ml dH<sub>2</sub>O, mix.
- 6. 10X Tris Buffered Saline with Tween® 20 (TBST):** (#9997) To prepare 1 L 1X TBST: add 100 ml 10X TBST to 900 ml dH<sub>2</sub>O, mix.
- 7. Nonfat Dry Milk:** (#9999)
- 8. Blocking Buffer:** 1X TBST with 5% w/v nonfat dry milk; for 150 ml, add 7.5 g nonfat dry milk to 150 ml 1X TBST and mix well.
- 9. Wash Buffer:** (#9997) 1X TBST
- 10. Bovine Serum Albumin (BSA):** (#9998)
- 11. Primary Antibody Dilution Buffer:** 1X TBST with 5% BSA or 5% nonfat dry milk as indicated on primary antibody datasheet; for 20 ml, add 1.0 g BSA or nonfat dry milk to 20 ml 1X TBST and mix well.
- 12. Biotinylated Protein Ladder Detection Pack:** (#7727)
- 13. Prestained Protein Marker, Broad Range (Premixed Format):** (#7720)
- 14. Blotting Membrane and Paper:** (#12369) This protocol has been optimized for nitrocellulose membranes. Pore size 0.2 µm is generally recommended.
- 15. Secondary Antibody Conjugated to HRP:** anti-rabbit (#7074); anti-mouse (#7076)
- 16. Detection Reagent:** LumiGLO® chemiluminescent reagent and peroxide (#7003) or SignalFire™ ECL Reagent (#6883)

### B. Protein Blotting

#### A general protocol for sample preparation.

1. Treat cells by adding fresh media containing regulator for desired time.
2. Aspirate media from cultures; wash cells with 1X PBS; aspirate.

3. Lyse cells by adding 1X SDS sample buffer (100 µl per well of 6-well plate or 500 µl for a 10 cm diameter plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.
4. Sonicate for 10–15 sec to complete cell lysis and shear DNA (to reduce sample viscosity).
5. Heat a 20 µl sample to 95–100°C for 5 min; cool on ice.
6. Microcentrifuge for 5 min.
7. Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm). **NOTE:** Loading of prestained molecular weight markers (#7720, 10 µl/lane) to verify electrotransfer and biotinylated protein ladder (#7727, 10 µl/lane) to determine molecular weights are recommended.
8. Electrotransfer to nitrocellulose membrane (#12369).

### C. Membrane Blocking and Antibody Incubations

**NOTE:** Volumes are for 10 cm x 10 cm (100 cm<sup>2</sup>) of membrane; for different sized membranes, adjust volumes accordingly.

#### I. Membrane Blocking

1. (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 min at room temperature.
2. Incubate membrane in 25 ml of blocking buffer for 1 hr at room temperature.
3. Wash three times for 5 min each with 15 ml of TBST.

#### II. Primary Antibody Incubation

Proceed to one of the following specific set of steps depending on the primary antibody used.

#### For Unconjugated Primary Antibodies

1. Incubate membrane and primary antibody (at the appropriate dilution and diluent as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
2. Wash three times for 5 min each with 15 ml of TBST.
3. Incubate membrane with the species appropriate HRP-conjugated secondary antibody (#7074 or #7076 at 1:2000) and anti-biotin, HRP-linked Antibody (#7075 at 1:1000–1:3000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 hr at room temperature.
4. Wash three times for 5 min each with 15 ml of TBST.
5. Proceed with detection (Section D).

#### For HRP Conjugated Primary Antibodies

1. Incubate membrane and primary antibody (at the appropriate dilution as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
2. Wash three times for 5 min each with 15 ml of TBST.
3. Incubate with Anti-biotin, HRP-linked Antibody (#7075 at 1:1000–1:3000), to detect biotinylated protein markers, in 10 ml of blocking buffer with gentle agitation for 1 hr at room temperature.
4. Wash three times for 5 min each with 15 ml of TBST.
5. Proceed with detection (Section D).

#### For Biotinylated Primary Antibodies

1. Incubate membrane and primary antibody (at the appropriate dilution as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
2. Wash three times for 5 min each with 15 ml of TBST.
3. Incubate membrane with Streptavidin-HRP (#3999 at the appropriate dilution) in 10 ml of blocking buffer with gentle agitation for 1 hr at room temperature.
4. Wash three times for 5 min each with 15 ml of TBST.
5. Proceed with detection (Section D).

**Do not add** Anti-biotin, HRP-linked Antibody for detection of biotinylated protein markers. There is no need. The Streptavidin-HRP will also visualize the biotinylated markers.

### D. Detection of Proteins

1. Incubate membrane with 10 ml LumiGLO® (0.5 ml 20X LumiGLO® #7003, 0.5 ml 20X Peroxide, and 9.0 ml purified water) or 10 ml SignalFire™ #6883 (5 ml Reagent A, 5 ml Reagent B) with gentle agitation for 1 min at room temperature.
2. Drain membrane of excess developing solution (do not let dry), wrap in plastic wrap and expose to x-ray film. An initial 10 sec exposure should indicate the proper exposure time. **NOTE:** Due to the kinetics of the detection reaction, signal is most intense immediately following incubation and declines over the following 2 hr.



# Western Immunoblotting (WB)

## Fluorescent

last updated: 8/21/13

For western blots, incubate membrane with diluted primary antibody in either 5% w/v BSA or nonfat dry milk, 1X TBS, 0.1% Tween® 20 at 4°C with gentle shaking, overnight. **NOTE:** Two-color western blots require primary antibodies from different species and appropriate secondary antibodies labeled with different dyes. Overlap of epitopes may cause interference and should be considered in two color western blots. If the primary antibodies require different primary antibody incubation buffers, test each primary individually in both buffers to determine the optimal one for the dual-labeling experiment.

### A. Solutions and Reagents

**NOTE:** Prepare solutions with reverse osmosis deionized (RODI) or equivalent grade water.

- 20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L 1X PBS: add 50 ml 20X PBS to 950 ml dH<sub>2</sub>O, mix.
- 10X Tris Buffered Saline (TBS):** (#12498) To prepare 1 L 1X TBS: add 100 ml 10X TBS to 900 ml dH<sub>2</sub>O, mix.
- 1X SDS Sample Buffer:** Blue Loading Pack (#7722) or Red Loading Pack (#7723) Prepare fresh 3X reducing loading buffer by adding 1/10 volume 30X DTT to 1 volume of 3X SDS loading budder. Dilute to 1X with dH<sub>2</sub>O.
- 10X Tris-Glycine SDS Running Buffer:** (#4050) To prepare 1 L 1X running buffer: add 100 ml 10X running buffer 900 ml dH<sub>2</sub>O, mix.
- 10X Tris-Glycine Transfer Buffer:** (#12539) To prepare 1 L 1X Transfer Buffer: add 100 ml 10X Transfer Buffer 200 ml methanol + 700 ml dH<sub>2</sub>O, mix.
- 10X Tris Buffered Saline with Tween® 20 (TBST-10X):** (#9997) To prepare 1 L 1X TBST: add 100 ml 10X TBST to 900 ml dH<sub>2</sub>O, mix.
- Nonfat Dry Milk:** (#9999)
- Blocking Buffer:** 1X TBS with 5% w/v nonfat dry milk; for 150 ml, add 7.5 g nonfat dry milk to 150 ml 1X TBS and mix well. Tween® 20 should not be present in the Blocking Buffer because it is auto-fluorescent and increases non-specific background. After the blocking step, Tween® 20 can be reintroduced to subsequent diluent buffers.
- Wash Buffer:** 1X TBST
- Bovine Serum Albumin (BSA):** (#9998)
- Primary Antibody Dilution Buffer:** 1X TBST with 5% BSA or 5% nonfat dry milk as indicated on primary antibody datasheet; for 20 ml, add 1.0 g BSA or nonfat dry milk to 20 ml 1X TBST and mix well.
- Secondary Antibody Dilution Buffer:** 1X TBST with 5% nonfat dry milk; for 20 ml, add 1.0 g nonfat dry milk to 20 ml 1X TBST and mix well. (Secondary antibodies; anti-rabbit #5151 and #5366; anti-mouse #5257 and #5470)
- Prestained Protein Marker, Broad Range (Premixed Format):** (#7720)
- Blotting Membrane and Paper:** (#12369) This protocol has been optimized for nitrocellulose membranes (recommended). Pore size 0.2 µm is generally recommended.

### B. Protein Blotting

**A general protocol for sample preparation.**

- Treat cells by adding fresh media containing regulator for desired time.
  - Aspirate media from cultures; wash cells with cold 1X PBS; aspirate.
  - Lyse cells by adding 1X SDS sample buffer (100 µl per well of 6-well plate or 500 µl per plate of 10 cm diameter plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.
  - Sonicate for 10–15 sec to complete cell lysis and shear DNA (to reduce sample viscosity).
  - Heat a 20 µl sample to 95–100°C for 5 min; cool on ice.
  - Microcentrifuge for 5 min.
  - Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm).
- NOTE:** Loading of prestained molecular weight markers (#7720, 10 µl/lane) is recommended to verify electrotransfer and to determine molecular weights. Prestained markers are autofluorescent at near-infrared wavelengths.
- Electrotransfer to nitrocellulose membrane (#12369).

### C. Membrane Blocking and Antibody Incubations

**NOTE:** Volumes are for 10 cm x 10 cm (100 cm<sup>2</sup>) of membrane; for different sized membranes, adjust volumes accordingly.

- (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 min at room temperature.
- Incubate membrane in 25 ml of blocking buffer for 1 hr at room temperature. **CRITICAL STEP:** Do not include Tween® 20 in blocking buffer (Section A, Step 8).
- Wash three times for 5 min each with 15 ml of TBST.
- Incubate membrane and primary antibody (at the appropriate dilution as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
- Wash three times for 5 min each with 15 ml of TBST.
- Incubate membrane with fluorophore-conjugated secondary antibody (#5470, #5257, #5366, #5151) (1:5000–1:25,000 dilution of 1 mg/ml stock) in 10 ml of secondary antibody dilution buffer with gentle agitation for 1 hr at room temperature.
- Wash three times for 5 min each with 15 ml of TBST.

### D. Detection of Proteins

- Drain membrane of excess TBST and allow to dry. **CRITICAL STEP:** Membrane must be dry for fluorescent staining.
- Scan membrane using an appropriate fluorescent scanner following the manufacturer's recommendations.

# Western Immunoblotting (WB)

## Troubleshooting Guide

last updated: 8/21/13

**HIGH BACKGROUND:** General background is high or nonspecific bands appear after a short exposure of blot to film. Be conscious of the fact that motif antibodies, post-translational modifications, and splice variants may result in multiple banding.

| Cause:   | Solution:   |
|--|---|
| <b>Lysate preparation</b>                            | Freshly prepared samples result in fewer nonspecific and degradation bands, and therefore yield a cleaner blot. In general, tissue extracts tend to contain more background bands and degradation products than cell line extracts due to connective tissue. Using fresh, sonicated and clarified tissue extracts may lessen background. Samples should always be lysed in appropriate buffers that include protease inhibitors and phosphatase inhibitors for phospho-targets (Phosphatase Inhibitor Cocktail (100X) #5870, Protease/Phosphatase Inhibitor Cocktail (100X) #5872, Protease Inhibitor Cocktail #5871). We recommend the use of Cell Lysis Buffer (#9803) or RIPA Buffer (#9806), which contains stronger detergent, for sample preparation and protein concentration quantification. SDS loading buffer (#7722 or #7723) may be used for whole cell lysis without protein quantification. Chaps Cell Extract Buffer (#9852) is used to prepare cytoplasmic cell fractions and is useful for studying caspase signaling. Cell Fractionation Kit (#9038) is used to prepare cytoplasmic, membrane/organelle, and nuclear/cytoskeletal cell fractions. |
| <b>Membrane</b>                                      | Use high quality nitrocellulose membrane (Nitrocellulose Sandwiches #12369). Pore size 0.2 $\mu$ m is generally recommended; membranes with a pore size of 0.45 $\mu$ m are not recommended for proteins smaller than 30 kDa. PVDF membranes may yield higher background than nitrocellulose. Nylon membranes are not recommended for western blotting. Block for 1 hr at room temperature in 5% nonfat dry milk in TBST.   |
| <b>Primary antibody dilution and/or incubation</b>   | Incubate primary antibody overnight at 4°C in TBST at the recommended dilution with the recommended blocking agent (either 5% BSA or 5% nonfat dry milk). For individual antibodies please consult the product datasheet for recommended dilution buffer.   |
| <b>Inadequate washing</b>                            | Washing for less time than the recommended three times 5 min in TBST is common, and can result in high background. We recommend that washes be timed to ensure accuracy. Additionally, low Tween® 20 can contribute to high background; we recommend 0.1% Tween® 20. This applies to washing steps after both primary and secondary antibody incubations.   |
| <b>Secondary antibody dilution and/or incubation</b> | Some secondary antibodies bind nonspecifically to proteins in cell extracts. To assess the quality of a secondary antibody, perform a blot (through to film exposure) without primary antibody. Serial dilutions of the secondary antibody can be performed on blots with the same cell extracts and primary antibody to optimize secondary antibody concentration. Always incubate the secondary antibody in 5% nonfat dry milk in TBST for 1 hr at room temperature, diluting the secondary antibody in BSA yields higher levels of background bands. CST™ secondary antibodies have already been optimized and do not require titration.   |
| <b>Detection reagent</b>                             | Chemiluminescent detection requires that high purity water be used to dilute concentrated reagents. Use only purified water with organic and inorganic impurities removed. We recommend using RODI water. Additionally, ensure that the membrane is never allowed to dry out during the antibody incubation process prior to detection reagent exposure.  |
| <b>Exposure time</b>                                 | Film exposure times of more than 30 sec lead to increased background signal. To avoid long exposure times, it is important to use cell lines or tissues with adequate protein expression levels and use recommended primary antibody incubation conditions (see primary antibody dilution and/or incubation section). If necessary, use treatment to induce expression or modification.   |

**LOW SIGNAL:** The protein of interest cannot be detected after a short exposure of blot to film.

| Cause:   | Solution:   |
|--|---|
| <b>Lysate preparation</b>                            | 20–30 $\mu$ g total protein from whole cell extracts per lane is usually sufficient for detection. If basal levels of target protein, or protein modification are low, it may be necessary to induce expression or modification via chemical stimulant. You may want to investigate alternative cell lines or tissues in which the protein of interest is more abundant. Samples should always be lysed in appropriate buffers that include protease inhibitors and phosphatase inhibitors, for phospho-targets (Phosphatase Inhibitor Cocktail (100X) #5870, Protease/Phosphatase Inhibitor Cocktail (100X) #5872, Protease Cocktail #5871). Lysates should always be sonicated to ensure efficient protein extraction of chromatin and membrane-bound targets. Please visit the Controls Table on our website for suggested positive controls and treatments. |
| <b>Primary antibody dilution and/or incubation</b>   | Incubate primary antibody overnight at 4°C in TBST at the recommended dilution with the recommended blocking agent (5% BSA or 5% nonfat dry milk). The use of alternate blocking agents, such as gelatin, serum, protein-free blocking agents, casein, or mixed blocking agents may reduce target signal intensity. For optimal results with individual antibodies, please consult the product datasheet for recommended dilution buffer.   |
| <b>SDS-PAGE Gel selection</b>                        | In general, Tris-Glycine gels are recommended. However, for high molecular weight proteins we recommend Tris-Acetate gels and associated buffers. Proteins transfer more efficiently from 1 mm gels than from 1.5 mm gels. The use of thicker gels can result in incomplete transfer of high molecular weight proteins, so we recommend monitoring transfer efficiency and implementing modifications of transfer conditions to optimize for the size of your protein target of interest.   |
| <b>Transfer and Membrane</b>                         | Incomplete transfer can be corrected by longer transfer or by the use of higher voltage. In general, we recommend including 20% methanol in transfer buffer, and performing a wet transfer for 1.5 hr at 70 V. For high molecular weight proteins, we recommend reducing the methanol in transfer buffer to 5% to improve transfer efficiency and avoid fixing large proteins in the gel matrix, as well as increasing transfer time to 3 hr at 70 V. Over transfer (protein transfer through membrane) can be problematic when examining smaller proteins. For small proteins, it is important to use a 0.2 $\mu$ m pore size membrane and wet transfer for 1.5 hr at 70 V, not a 0.45 $\mu$ m pore size membrane or transferring overnight, which may result in over transfer and a lack of small protein signal.   |
| <b>Blocking</b>                                      | Blocking the membrane for too long can obscure antigenic epitopes and prevent the antibody from binding. Block for only 1 hr at room temperature.   |
| <b>Washing</b>                                       | Washing for longer than the recommended three times 5 min is a common issue and can result in reduced signal. We recommend that washes be timed to ensure accuracy. TBST should contain 0.1% Tween® 20. This applies to washing steps after both primary and secondary antibody incubations. Additionally, we recommend washing in TBST, as washing in PBST has been shown to result in reduced signal.   |
| <b>Secondary antibody dilution and/or incubation</b> | Serial dilutions of the secondary antibody can be performed on blots with the same cell extracts and primary antibody to optimize secondary antibody concentration. Always incubate the secondary antibody in 5% nonfat dry milk in TBST for 1 hr at room temperature. Avoid including sodium azide in HRP-conjugated secondary antibody buffers as azide inhibits HRP activity. CST™ secondary antibodies have already been optimized and do not require titration.  |
| <b>Detection reagent</b>                             | Use biotinylated molecular weight standards that can be detected with anti-biotin-HRP as positive controls for chemiluminescent detection.  |



# Immunoprecipitation (IP)

## Native Proteins

last updated: 8/21/13

This protocol is intended for immunoprecipitation of native proteins for analysis by western immunoblotting or kinase activity.

### A. Solutions and Reagents

**NOTE:** Prepare solutions with reverse osmosis deionized (RODI) or equivalent grade water.

- 1. 20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L of 1X PBS, add 50 ml 20X PBS to 950 ml dH<sub>2</sub>O, mix.
- 2. 10X Cell Lysis Buffer:** (#9803) To prepare 10 ml of 1X cell lysis buffer, add 100 ml cell lysis buffer to 9 ml dH<sub>2</sub>O, mix. **NOTE:** Add 1 mM PMSF (#8553) immediately prior to use.
- 3. 3X SDS Sample Buffer:** Blue Loading Pack (#7722) or Red Loading Pack (#7723) Prepare fresh 3X reducing loading buffer by adding 1/10 volume 30X DTT to 1 volume of 3X SDS loading buffer.
- 4. Protein A or G Agarose Beads (For unconjugated primary antibodies):** Use Protein A (#9863, #8687) for rabbit IgG immunoprecipitation and Protein G (#8740) for mouse IgG immunoprecipitation. **NOTE:** Magnetic beads (#8687, #8740) require 6-Tube Magnetic Separation Rack (#7017).
- 5. Immobilized Streptavidin (Bead Conjugate) (For biotinylated antibodies):** (#3419) Gently vortex vial and use 10 µl per immunoprecipitation.
- 6. 10X Kinase Buffer (for kinase assays):** (#9802) To Prepare 1 ml of 1X kinase buffer, add 100 µl 10X kinase buffer to 900 µl dH<sub>2</sub>O, mix.
- 7. ATP (10 mM) (for kinase assays):** (#9804) To prepare 0.5 ml of ATP (200 µM), add 10 µl ATP (10 mM) to 490 µl 1X kinase buffer.

### B. Preparing Cell Lysates

- Aspirate media. Treat cells by adding fresh media containing regulator for desired time.
- To harvest cells under nondenaturing conditions, remove media and rinse cells once with ice-cold 1X PBS.
- Remove PBS and add 0.5 ml ice-cold 1X cell lysis buffer to each plate (10 cm) and incubate on ice for 5 min.
- Scrape cells off the plate and transfer to microcentrifuge tubes. Keep on ice.
- Sonicate on ice three times for 5 sec each.
- Microcentrifuge for 10 min at 4°C, 14,000 x g and transfer the supernatant to a new tube. The supernatant is the cell lysate. If necessary, lysate can be stored at -80°C.

### C. Immunoprecipitation

#### Cell Lysate Pre-Clearing

##### (Optional step for unconjugated and biotinylated antibodies.)

- Add 10–30 µl of 50% bead slurry, either Protein A or G agarose or magnetic beads (for unconjugated primary antibodies) or 10 µl streptavidin beads (#3419; for biotinylated antibodies), to 200 µl cell lysate at 1 mg/ml.
- Incubate with rotation at 4°C for 30–60 min.
- Microcentrifuge for 10 min at 4°C. Transfer the supernatant to a fresh tube.
- Proceed to one of the following specific set of steps depending on the primary antibody used.

#### Using Unconjugated Primary Antibodies

- Add primary antibody (at the appropriate dilution as recommended in the product datasheet) to 200 µl cell lysate at 1 mg/ml. Incubate with gentle rocking overnight at 4°C.
- Add either protein A or G agarose or magnetic beads (10–30 µl of 50% bead slurry). Incubate with gentle rocking for 1–3 hr at 4°C for agarose beads, or 10–30 min for magnetic beads.
- Microcentrifuge for 30 sec at 4°C. Wash pellet five times with 500 µl of 1X cell lysis buffer. Keep on ice between washes.
- Proceed to analyze by western immunoblotting or kinase activity (Section D).

#### Using Biotinylated Primary Antibodies

- Add biotinylated antibody (at the appropriate dilution as recommended in the product datasheet) to 200 µl cell lysate at 1 mg/ml. Incubate with gentle rocking overnight at 4°C.
- Gently mix Immobilized Streptavidin (Sephacrose® Bead Conjugate #3419) and add 10 µl of slurry. Incubate with gentle rocking for 2 hr at 4°C.
- Microcentrifuge for 30 sec at 4°C. Wash pellet five times with 500 µl of 1X cell lysis buffer. Keep on ice during washes.
- Proceed to analyze by western immunoblotting or kinase activity (Section D).

#### Using Immobilized Antibodies (Sephacrose® Bead Conjugate)

- Vortex gently and add immobilized bead conjugate (10 µl) to 200 µl cell lysate at 1 mg/ml. Incubate with gentle rocking overnight at 4°C.
- Microcentrifuge for 30 sec at 4°C. Wash pellet five times with 500 µl of 1X cell lysis buffer. Keep on ice during washes.
- Proceed to analyze by western immunoblotting or kinase activity (Section D).

#### Using Immobilized Antibodies (Magnetic Bead Conjugate)

- Vortex gently and add immobilized bead conjugate (10 µl) to 200 µl cell lysate at 1 mg/ml. Incubate with gentle rocking overnight at 4°C.
- Pellet magnetic beads by placing the tubes in a magnetic separation rack (#7017) and wait 1 to 2 min for solution to clear. Wash pellet five times with 500 µl of 1X cell lysis buffer. Keep on ice during washes.
- Proceed to analyze by western immunoblotting or kinase activity (Section D).

### D. Sample Analysis

Proceed to one of the following specific set of steps. **NOTE:** For magnetic beads, do not centrifuge. Instead use a magnetic separation rack (#7017).

#### For Analysis by Western Immunoblotting

- Resuspend the pellet with 20 µl 3X SDS sample buffer. Vortex, then microcentrifuge for 30 sec.
- Heat the sample to 95–100°C for 2–5 min and microcentrifuge for 1 min at 14,000 x g.
- Load the sample (15–30 µl) on a 4–20% gel for SDS-PAGE.
- Analyze sample by western blot (see Western Immunoblotting Protocol).

**NOTE:** For proteins with molecular weights near 50 kDa, we recommend using Mouse Anti-rabbit IgG (Light-Chain Specific) (L57A3) mAb #3677 or Mouse Anti-rabbit IgG (Conformation Specific) (L27A9) mAb #3678 as a secondary antibody to minimize masking produced by denatured heavy chains. For proteins with molecular weights near 25 kDa, Mouse Anti-rabbit IgG (Conformation Specific) (L27A9) mAb #3678 or Mouse Anti-rabbit IgG (Conformation Specific) (L27A9) mAb (HRP Conjugate) #5127 is recommended.

#### For Analysis by Kinase Assay

- Wash pellet twice with 500 µl 1X kinase buffer. Keep on ice.
- Suspend pellet in 40 µl 1X kinase buffer supplemented with 200 µM ATP and appropriate substrate.
- Incubate for 30 min at 30°C.
- Terminate reaction with 20 µl 3X SDS sample buffer. Vortex, then microcentrifuge for 30 sec.
- Transfer supernatant containing phosphorylated substrate to another tube.
- Heat the sample to 95–100°C for 2–5 min and microcentrifuge for 1 min at 14,000 x g.
- Load the sample (15–30 µl) on SDS-PAGE (4–20%).

# Immunohistochemistry (IHC)

## Paraffin (using SignalStain® Boost Detection Reagent)

last updated: 8/21/13

**\*IMPORTANT:** Please refer to the APPLICATIONS section on the front page of product datasheet or product webpage to determine whether a product is validated and approved for use on paraffin-embedded (IHC-P) tissue sections. Please see product datasheet or product webpage for appropriate antibody dilution and unmasking solution.

### A. Solutions and Reagents

**NOTE:** Prepare solutions with reverse osmosis deionized (RODI) or equivalent grade water.

1. Xylene
2. Ethanol, anhydrous denatured, histological grade (100% and 95%)
3. Deionized water (dH<sub>2</sub>O)
4. Hematoxylin (optional)
5. **Wash Buffer:**
  - a. **10X Tris Buffered Saline with Tween® 20 (TBST):** (#9997) To prepare 1 L 1X TBST: add 100 ml 10X TBST to 900 ml dH<sub>2</sub>O, mix.
6. **\*Antibody Diluent Options:**
  - a. **SignalStain® Antibody Diluent:** (#8112)
  - b. **TBST/5% normal goat serum:** To 5 ml 1X TBST, add 250 µl Normal Goat Serum (#5425).
  - c. **PBST/5% normal goat serum:** To 5 ml 1X PBST, add 250 µl Normal Goat Serum (#5425).

**20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L 1X PBS: add 50 ml 20X PBS to 950 ml dH<sub>2</sub>O, mix.

**1X PBS/0.1% Tween® 20 (1X PBST):** To prepare 1 L 1X PBST, add 100 ml 10X PBS to 900 ml dH<sub>2</sub>O. Add 1 ml Tween® 20 and mix.
7. **\*Antigen Unmasking Options:**
  - a. **Citrate:** 10 mM Sodium Citrate Buffer: To prepare 1 L, add 2.94 g sodium citrate trisodium salt dihydrate (C<sub>6</sub>H<sub>5</sub>Na<sub>3</sub>O<sub>7</sub>•2H<sub>2</sub>O) to 1 L dH<sub>2</sub>O. Adjust pH to 6.0.
  - b. **EDTA:** 1 mM EDTA: To prepare 1 L add 0.372 g EDTA (C<sub>10</sub>H<sub>14</sub>N<sub>2</sub>O<sub>8</sub>Na<sub>2</sub>•2H<sub>2</sub>O) to 1 L dH<sub>2</sub>O. Adjust pH to 8.0.
  - c. **TE:** 10 mM Tris/1 mM EDTA, pH 9.0: To prepare 1 L, add 1.21 g Tris base (C<sub>4</sub>H<sub>11</sub>NO<sub>3</sub>) and 0.372 g EDTA (C<sub>10</sub>H<sub>14</sub>N<sub>2</sub>O<sub>8</sub>Na<sub>2</sub>•2H<sub>2</sub>O) to 950 ml dH<sub>2</sub>O. Adjust pH to 9.0, then adjust final volume to 1 L with dH<sub>2</sub>O.
  - d. **Pepsin:** 1 mg/ml in Tris-HCl, pH 2.0.
8. **3% Hydrogen Peroxide:** To prepare 100 ml, add 10 ml 30% H<sub>2</sub>O<sub>2</sub> to 90 ml dH<sub>2</sub>O.
9. **Blocking Solution:** TBST/5% Normal Goat Serum: to 5 ml 1X TBST, add 250 µl Normal Goat Serum (#5425).
10. **Detection System:** SignalStain® Boost IHC Detection Reagents (HRP, Mouse #8125; HRP, Rabbit #8114)
11. **Substrate:** SignalStain® DAB Substrate Kit (#8059).

### B. Deparaffinization/Rehydration

**NOTE:** Do not allow slides to dry at any time during this procedure.

1. **Deparaffinize/hydrate sections:**
  - a. Incubate sections in three washes of xylene for 5 min each.
  - b. Incubate sections in two washes of 100% ethanol for 10 min each.
  - c. Incubate sections in two washes of 95% ethanol for 10 min each.
2. Wash sections two times in dH<sub>2</sub>O for 5 min each.

### C. Antigen Unmasking\*

**NOTE:** Consult product datasheet for specific recommendation for the unmasking solution/protocol.

1. **For Citrate:** Bring slides to a boil in 10 mM sodium citrate buffer, pH 6.0; maintain at a sub-boiling temperature for 10 min. Cool slides on bench top for 30 min.
2. **For EDTA:** Bring slides to a boil in 1 mM EDTA, pH 8.0; follow with 15 min at a sub-boiling temperature. No cooling is necessary.
3. **For TE:** Bring slides to a boil in 10 mM Tris/1 mM EDTA, pH 9.0; then maintain at a sub-boiling temperature for 18 min. Cool at room temperature for 30 min.
4. **For Pepsin:** Digest for 10 min at 37°C.

### D. Staining

**NOTE:** Consult product datasheet for recommended antibody diluent.

1. Wash sections in dH<sub>2</sub>O three times for 5 min each.
2. Incubate sections in 3% hydrogen peroxide for 10 min.
3. Wash sections in dH<sub>2</sub>O two times for 5 min each.
4. Wash sections in wash buffer for 5 min.
5. Block each section with 100–400 µl blocking solution for 1 hr at room temperature.
6. Remove blocking solution and add 100–400 µl primary antibody diluted in recommended antibody diluent to each section\*. Incubate overnight at 4°C.
7. Equilibrate SignalStain® Boost Detection Reagent to room temperature.
8. Remove antibody solution and wash sections with wash buffer three times for 5 min each.
9. Cover section with 1–3 drops SignalStain® Boost Detection Reagent as needed. Incubate in a humidified chamber for 30 min at room temperature.
10. Wash sections three times with wash buffer for 5 min each.
11. Add 1 drop (30 µl) SignalStain® DAB Chromogen Concentrate to 1 ml SignalStain® DAB Diluent and mix well before use.
12. Apply 100–400 µl SignalStain® DAB to each section and monitor closely. 1–10 min generally provides an acceptable staining intensity.
13. Immerse slides in dH<sub>2</sub>O.
14. If desired, counterstain sections with hematoxylin per manufacturer's instructions.
15. Wash sections in dH<sub>2</sub>O two times for 5 min each.
16. **Dehydrate sections:**
  - a. Incubate sections in 95% ethanol two times for 10 sec each.
  - b. Repeat in 100% ethanol, incubating sections two times for 10 sec each.
  - c. Repeat in xylene, incubating sections two times for 10 sec each.
17. Mount sections with coverslips.





# Immunohistochemistry (IHC)

last updated: 8/21/13

## Frozen (using SignalStain® Boost Detection Reagent)

**\*IMPORTANT:** Please refer to the APPLICATIONS section on the front page of product datasheet or product webpage to determine whether a product is validated and approved for use frozen tissue sections. Please see product datasheet or product webpage for appropriate antibody dilution and unmasking solution. **NOTE:** Please see product datasheet and website for product-specific protocol recommendations.

### A. Solutions and Reagents

**NOTE:** Prepare solutions with reverse osmosis deionized (RODI) or equivalent grade water.

1. Xylene
2. Ethanol (anhydrous denatured, histological grade 100% and 95%)
3. Hematoxylin (optional)
4. **20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L 1X PBS: add 50 ml 20X PBS to 950 ml dH<sub>2</sub>O, mix.
5. **Fixative Options:** For optimal fixative, please refer to the product datasheet.
  - a. 10% neutral buffered formalin
  - b. Acetone
  - c. Methanol
  - d. **3% formaldehyde:** To prepare 100 ml, add 18.75 ml 16% formaldehyde to 81.25 ml 1X PBS.
6. **10X Tris Buffered Saline (TBS) Wash Buffer:** (#12498) To prepare 1 L 1X TBS add 100 ml of 10X TBS to 900 ml dH<sub>2</sub>O, mix.
7. **Methanol/Peroxidase:** To prepare, add 10 ml 30% H<sub>2</sub>O<sub>2</sub> to 90 ml methanol. Store at -20°C.
8. **Blocking Solution:** 1X TBS/0.3% Triton™ X-100/5% Normal Goat Serum (#5425). To prepare, add 500 µl goat serum and 30 µl Triton™ X-100 to 9.5 ml 1X TBS.
9. **Detection System:** SignalStain® Boost IHC Detection Reagents (HRP, Mouse #8125; HRP, Rabbit #8114)
10. **Substrate:** SignalStain® DAB Substrate Kit (#8059).

### B. Sectioning

1. **For tissue stored at -80°C:** Remove from freezer and equilibrate at -20°C for approximately 15 min before attempting to section. This may prevent cracking of the block when sectioning.
2. Section tissue at a range of 6–8 µm and place on positively charged slides.
3. Allow sections to air dry on bench for a few min before fixing (this helps sections adhere to slides).

### C. Fixation Options

**NOTE:** Consult product datasheet to determine the optimal fixative.

1. After sections have dried on the slide, fix in optimal fixative as directed below.
  - a. **10% Neutral buffered formalin:** 10 min at room temperature. Proceed with staining procedure immediately (Section D).
  - b. **Cold acetone:** 10 min at -20°C. Air dry. Proceed with staining immediately (Section D).
  - c. **Methanol:** 10 min at -20°C. Proceed with staining immediately (Section D).
  - d. **3% Formaldehyde:** 15 min at room temperature. Proceed with staining immediately (Section D).
  - e. **3% Formaldehyde/methanol:** 15 min at room temperature in 3% formaldehyde, followed by 5 min in methanol at -20°C (do not rinse in between). Proceed with staining immediately (Section D).

### D. Staining

1. Wash sections in wash buffer two times for 5 min.
2. Incubate for 10 min at room temperature in methanol/peroxidase.
3. Wash sections in wash buffer two times for 5 min.
4. Block each section with 100–400 µl blocking solution for 1 hr at room temperature.
5. Remove blocking solution and add 100–400 µl primary antibody diluted in blocking solution to each section.
6. Incubate overnight at 4°C.
7. Equilibrate SignalStain® Boost Detection Reagent to room temperature.
8. Remove antibody solution and wash sections in wash buffer three times for 5 min each.
9. Cover section with 1–3 drops SignalStain® Boost Detection Reagent as needed. Incubate in a humidified chamber for 30 min at room temperature.
10. Wash sections three times with wash buffer for 5 min each.
11. Add 1 drop (30 µl) SignalStain® DAB Chromogen Concentrate to 1 ml SignalStain® DAB Diluent and mix well before use.
12. Apply 100–400 µl SignalStain® DAB to each section and monitor closely. 1–10 min generally provides an acceptable staining intensity.
13. Immerse slides in dH<sub>2</sub>O.
14. If desired, counterstain sections with hematoxylin per manufacturer's instructions.
15. Wash sections in dH<sub>2</sub>O two times for 5 min each.
16. **Dehydrate sections:**
  - a. Incubate sections in 95% ethanol two times for 10 sec each.
  - b. Repeat in 100% ethanol, incubating sections two times for 10 sec each.
  - c. Repeat in xylene, incubating sections two times for 10 sec each.
17. Mount sections with coverslips.



# Immunohistochemistry (IHC)

## Troubleshooting Guide

last updated: 8/21/13

Suboptimal IHC staining is frequently resolved by adjusting relatively few variables. Adjustments to key steps within the protocol, such as antigen retrieval, can often resolve these common issues.

### LITTLE OR NO STAINING:

| Cause:                             | Solution:   |
|------------------------------------|---|
| <b>Sample storage</b>              | Slides may lose signal over time in storage. This process is variable and dependent upon the protein target. The effect of slide storage on staining has not been established for every protein; therefore, it is best practice that slides are freshly cut before use. If slides must be stored, do so at 4°C. Do not bake slides before storage.  |
| <b>Tissue sections dried out</b>   | It is vital that the tissue sections remain covered in liquid throughout the staining procedure.  |
| <b>Slide preparation</b>           | Inadequate deparaffinization may cause spotty, uneven background staining. Repeat the experiment with new sections using fresh xylene.  |
| <b>Antigen unmasking/retrieval</b> | Fixed tissue sections have chemical crosslinks between proteins that, dependent on the tissue and antigen target, may prevent antibody access or mask antigen targets. Antigen unmasking protocols may utilize a hot water bath, microwave, or pressure cooker. Antigen unmasking protocols utilizing a water bath are not recommended. Antigen unmasking performed with a microwave is preferred, though staining of particular tissues or antigen targets may require the use of a pressure cooker. |
| <b>Unmasking/retrieval buffer</b>  | Staining of particular tissues or antigen targets may require an optimized unmasking buffer. Refer to product datasheet for antigen unmasking buffer recommendations. Always prepare fresh 1X solutions daily.  |
| <b>Antibody dilution/diluent</b>   | Consult CST™ product datasheet for the recommended dilution and diluent. Titration of the antibody may be required if a reagent other than the one recommended is used.   |
| <b>Incubation time</b>             | Primary antibody incubation according to a rigorously tested protocol provides consistent, reliable results. CST™ antibodies have been developed and validated for optimal results when incubated overnight at 4°C.   |
| <b>Detection system</b>            | Polymer-based detection reagents, such as SignalStain® Boost IHC Detection Reagents (#8114) and (#8125), in conjunction with SignalStain® DAB Substrate Kit (#8059), are more sensitive than avidin/biotin-based detection systems. Standard secondary antibodies directly conjugated with HRP may not provide sufficient signal amplification. Always verify the expiration date of the detection reagent prior to use.  |
| <b>Negative staining</b>           | A complete lack of staining may indicate an issue with the antibody or protocol. Employ a high expressing positive control, such as paraffin-embedded cell pellets, to ensure that the antibody and procedure are working as expected. Phospho-specific antibodies in particular, or any antibody directed against a rarely expressed protein, may not stain 100% of the cases of a given indication. It is possible that the sample is truly negative.   |

### HIGH BACKGROUND:

| Cause:                            | Solution:   |
|-----------------------------------|---|
| <b>Slide preparation</b>          | Inadequate deparaffinization may cause spotty, uneven background staining. Repeat the experiment with new sections using fresh xylene.  |
| <b>Peroxidase quenching</b>       | Endogenous peroxidase activity in samples may produce excess background signal if an HRP-based detection system is being used. Quench slides in a 3% H <sub>2</sub> O <sub>2</sub> solution, diluted in RODI water, for 10 min prior to incubation with the primary antibody.   |
| <b>Biotin block</b>               | Using biotin-based detection systems with samples that have high levels of endogenous biotin, such as kidney and liver tissues, may be problematic. In this case, use a polymer-based detection system such as SignalStain® Boost IHC Detection Reagents (#8114) and (#8125). A biotin block may also be performed after the normal blocking procedure prior to incubation in primary antibody. |
| <b>Blocking</b>                   | Block slides with 1X TBS (#9997) with 5% Normal Goat Serum (#5425) for 30 min prior to incubation with the primary antibody.  |
| <b>Antibody dilution/diluent</b>  | Consult CST™ product datasheet for the recommended dilution and diluent. Titration of the antibody may be required if a reagent other than the one recommended is used.   |
| <b>Secondary cross reactivity</b> | The secondary antibody may bind endogenous IgG, causing high background, in some samples where the secondary antibody is raised in the same species as the sample being tested (mouse-on-mouse staining). Include a control slide stained without the primary antibody to confirm whether the secondary antibody is the source of the background.   |
| <b>Washes</b>                     | Adequate washing is critical for contrasting low background and high signal. Wash slides three times for 5 min with TBST (#9997) after primary and secondary antibody incubations.  |



# Notes:

# Flow Cytometry (F)

## General

last updated: 8/21/13

### A. Solutions and Reagents

**NOTE:** Prepare solutions with reverse osmosis deionized (RODI) or equivalent grade water.

1. **20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L 1X PBS: add 50 ml 20X PBS to 950 ml dH<sub>2</sub>O, mix.
2. **16% Formaldehyde** (methanol free)
3. **100% methanol**
4. **Incubation Buffer:** Dissolve 0.5 g Bovine Serum Albumin (BSA) (#9998) in 100 ml 1X PBS. Store at 4°C.
5. **Secondary Antibodies:** Anti-mouse (#4408, #8890, #4410, #8887). Anti-rabbit (#4412, #8889, #4414, #8885), Anti-rat (#4416, #4418)

### B. Fixation

1. Collect cells by centrifugation and aspirate supernatant.
2. Resuspend cells briefly in 0.5–1 ml 1X PBS. Add formaldehyde to a final concentration of 4% formaldehyde.
3. Fix for 10 min at 37°C.
4. Chill tubes on ice for 1 min.
5. For **extracellular staining** with antibodies that do not require permeabilization, proceed to immunostaining (Section D) or store cells in PBS with 0.1% sodium azide at 4°C; for **intracellular staining**, proceed to permeabilization (Section C).

### C. Permeabilization

**NOTE:** This step is critical for many GST antibodies.

1. Permeabilize cells by adding ice-cold 100% methanol slowly to pre-chilled cells, while gently vortexing, to a final concentration of 90% methanol. Alternatively, remove fix prior to permeabilization by centrifugation and resuspension in 90% methanol.
2. Incubate 30 min on ice.
3. Proceed with immunostaining (Section D) or store cells at -20°C in 90% methanol.

### D. Immunostaining

**NOTE:** Account for isotype matched controls for monoclonal antibodies or species matched IgG for polyclonal antibodies. Count cells using a hemocytometer or alternative method.

1. Aliquot 0.5–1x10<sup>6</sup> cells into each assay tube (by volume).
2. Add 2–3 ml incubation buffer to each tube and rinse by centrifugation. Repeat.
3. Resuspend cells in 100 µl incubation buffer per assay tube.
4. Block in Incubation Buffer for 10 min at room temperature.
5. Add the unconjugated, biotinylated, or fluorochrome-conjugated primary antibody at the appropriate dilution to the assay tubes (see individual antibody datasheet or product webpage for the appropriate dilution).
6. Incubate for 1 hr at room temperature.
7. Wash by centrifugation in 2–3 ml incubation buffer.
8. If using a fluorochrome-conjugated primary antibody, resuspend cells in 0.5 ml 1X PBS and analyze on flow cytometer; for unconjugated or biotinylated primary antibodies, proceed to immunostaining (Step 9).
9. Resuspend cells in fluorochrome-conjugated secondary antibody or fluorochrome-conjugated avidin, diluted in incubation buffer at the recommended dilution.
10. Incubate for 30 min at room temperature.
11. Wash by centrifugation in 2–3 ml incubation buffer.
12. Resuspend cells in 0.5 ml PBS and analyze on flow cytometer; alternatively, for DNA staining, proceed to optional DNA stain (Section E).

### E. Optional DNA Dye

1. Resuspend cells in 0.5 ml of DNA dye (e.g. Propidium Iodide (PI)/RNase Staining Solution #4087).
2. Incubate for at least 30 min at room temperature.
3. Analyze cells in DNA staining solution on flow cytometer.



# Flow Cytometry (F)

last updated: 8/21/13

## Alternate (for combined staining of intracellular proteins and cell surface markers in blood)

### A. Solutions and Reagents

**NOTE:** Prepare solutions with reverse osmosis deionized (RODI) or equivalent grade water.

- 1. 20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L 1X PBS: add 50 ml 20X PBS to 950 ml dH<sub>2</sub>O, mix.
- 2. 16% Formaldehyde** (methanol free)
- 3. Triton™ X-100:** To prepare 50 ml of 0.1% Triton™ X-100 add 25 ml Triton™ X-100 to 50 ml 1 X PBS and mix well.
- 4. 50% methanol**
- 5. Incubation Buffer:** Dissolve 0.5 g Bovine Serum Albumin (BSA) (#9998) in 100 ml 1X PBS. Store at 4°C.
- 6. Secondary Antibodies:** Anti-mouse (#4408, #8890, #4410, #8887). Anti-rabbit (#4412, #8889, #4414, #8885). Anti-rat (#4416, #4418)

### B. Preparation of Whole Blood (fixation, lysis, and permeabilization) for Immunostaining

1. Aliquot 100 µl fresh whole blood per assay tube.
- 2. OPTIONAL:** Place tubes in rack in 37°C water bath for short-term treatments with ligands, inhibitors, drugs, etc.
3. Add 65 µl of 10% formaldehyde to each tube.
4. Vortex briefly and let stand for 15 min at room temperature.
5. Add 1 ml of 0.1% Triton™ X-100 to each tube.
6. Vortex and let stand for 30 min at room temperature.
7. Add 1 ml incubation buffer.
8. Pellet cells by centrifugation and aspirate supernatant.
9. Repeat steps 7 and 8.
10. Resuspend cells in ice-cold 50% methanol in PBS (store methanol solution at -20°C until use).
11. Incubate at least 10 min on ice.
12. Proceed with staining or store cells at -20°C in 50% methanol.

### C. Staining Using Unlabeled Primary and Conjugated Secondary Antibodies

**NOTE:** Account for isotype-matched controls for monoclonal antibodies or species matched IgG for polyclonal antibodies.

1. Add 2–3 ml incubation buffer to each tube and rinse by centrifugation. Repeat.
2. Add primary antibodies diluted as recommended on datasheet or product webpage in incubation buffer.
3. Incubate for 30–60 min at room temperature.
4. Wash by centrifugation in 2–3 ml incubation buffer.
5. Resuspend cells in fluorochrome-conjugated secondary antibody diluted in incubation buffer according to the manufacturer's recommendations.
6. Incubate for 30 min at room temperature.
7. Wash by centrifugation in 2–3 ml incubation buffer.
8. Resuspend cells in 0.5 ml PBS and analyze on flow cytometer.

**Reference:** Chow, S., Hedley, D., Grom, P., Magari, R., Jacobberger, J.W., Shankey, T.V. (2005) Whole blood fixation and permeabilization protocol with red blood cell lysis for flow cytometry of intracellular phosphorylated epitopes in leukocyte subpopulations. *Cytometry A* 67, 4–17.



# Immunofluorescence (IF)

## General

last updated: 8/21/13

**IMPORTANT:** Please refer to the APPLICATIONS section on the front page of product datasheet or product webpage to determine if this product is validated and approved for use on cultured cell lines (IF-IC), paraffin-embedded samples (IF-P), or frozen tissue sections (IF-F). Please see product datasheet or product webpage for appropriate antibody dilution and unmasking solution. This protocol is recommended for both unconjugated and fluorophore conjugated antibodies. **NOTE:** Some CST™ antibodies work optimally using an alternate protocol. Please see product datasheet for product-specific recommendations.

### A. Solutions and Reagents

**NOTE:** Prepare solutions with reverse osmosis deionized (RODI) or equivalent grade water.

- 20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L 1X PBS: add 50 ml 20X PBS to 950 ml dH<sub>2</sub>O, mix.  
**NOTE:** Adjust pH to 8.0.
- Formaldehyde:** 16%, methanol free, Polysciences, Inc. (cat# 18814), use fresh, store opened vials at 4°C in dark. Dilute 1 in 4 in 1X PBS to make a 4% formaldehyde solution.
- Blocking Buffer:** (1X PBS/5% normal serum/0.3% Triton™ X-100): To prepare 10 ml, add 0.5 ml normal serum from the same species as the secondary antibody (e.g., Normal Goat Serum (#5425) to 9 ml 1X PBS) and mix well. While stirring, add 30 µl Triton™ X-100.
- Antibody Dilution Buffer:** (1X PBS/1% BSA/0.3% Triton™ X-100): To prepare 10 ml, add 30 µl Triton™ X-100 to 10 ml 1X PBS. Mix well then add 0.1g BSA (#9998), mix.
- Fluorochrome-conjugated Secondary Antibodies:** (Anti-mouse #4408, #4409, #8890, #4410) (Anti-rabbit #4412, #4413, #8889, #4414) (Anti-rat #4416, #4417, #4418)
- Prolong® Gold Antifade Reagent (#9071), Prolong® Gold Antifade Reagent with DAPI (#8961)

#### Reagents specific to IF-P application:

- Xylene
- Ethanol, anhydrous denatured, histological grade, 100% and 95%.
- Antigen Unmasking:**
  - For Citrate:** 10 mM Sodium Citrate Buffer: To prepare 1 L add 2.94 g (C<sub>6</sub>H<sub>5</sub>Na<sub>3</sub>O<sub>7</sub>•2H<sub>2</sub>O) to 1 L dH<sub>2</sub>O. Adjust pH to 6.0.
  - For EDTA:** 1 mM EDTA: To prepare 1 L add 0.372 g EDTA (C<sub>10</sub>H<sub>14</sub>N<sub>2</sub>O<sub>8</sub>Na<sub>2</sub>•2H<sub>2</sub>O) to 1 L dH<sub>2</sub>O. Adjust pH to 8.0.

### B. Specimen Preparation

#### I. Cultured Cell Lines (IF-IC)

**NOTE:** Cells should be grown, treated, fixed and stained directly in multi-well plates, chamber slides or on coverslips.

- Aspirate liquid, then cover cells to a depth of 2–3 mm with 4% formaldehyde diluted in warm PBS.  
**NOTE:** Formaldehyde is toxic, use only in a fume hood.
- Allow cells to fix for 15 min at room temperature.
- Aspirate fixative, rinse three times in 1X PBS for 5 min each.
- Proceed with Immunostaining (Section C).

#### II. Paraffin Sections (IF-P)

**NOTE:** Do not allow slides to dry at any time during this process.

- Deparaffinization/Rehydration:**
  - Wash three times in xylene for 5 min each.
  - Wash two times in 100% ethanol for 10 min each.

- Wash two times in 95% ethanol for 10 min each.
- Rinse sections two times in dH<sub>2</sub>O for 5 min each.

**2. Antigen Unmasking: NOTE:** Consult product datasheet or product webpage for specific recommendation for the unmasking solution.

- For Citrate:** Bring slides to a boil in 10 mM sodium citrate buffer pH 6.0, then maintain at a sub-boiling temperature for 10 min. Cool slides on bench top for 30 min.
- For EDTA:** Bring slides to a boil in 1 mM EDTA pH 8.0 followed by 15 min at a sub-boiling temperature. No cooling is necessary.

**3.** Proceed with Immunostaining (Section C).

#### III. Frozen/Cryostat Sections (IF-F)

- For fixed frozen tissue proceed with Immunostaining (Section C).
- For fresh, unfixed frozen tissue, fix immediately, as follows:
  - Cover sections with 4% formaldehyde diluted in warm 1X PBS.
  - Allow sections to fix for 15 min at room temperature.
  - Rinse slides three times in PBS for 5 min each.
- Proceed with Immunostaining (Section C).

### C. Immunostaining

**NOTE:** All subsequent incubations should be carried out at room temperature unless otherwise noted in a humid light-tight box or covered dish/plate to prevent drying and fluorochrome fading.

- Block specimen in blocking buffer for 60 min.
- While blocking, prepare primary antibody by diluting as indicated on datasheet in antibody dilution buffer.
- Aspirate blocking solution, apply diluted primary antibody.
- Incubate overnight at 4°C.
- Rinse three times in 1X PBS for 5 min each. **NOTE:** If using a fluorochrome-conjugated primary antibody, then skip to Section C, Step 8.
- Incubate specimen in fluorochrome-conjugated secondary antibody diluted in antibody dilution buffer for 1–2 hr at room temperature in the dark.
- Rinse three times in 1X PBS for 5 min each.
- Coverslip slides with Prolong® Gold Antifade Reagent (#9071) or Prolong® Gold Antifade Reagent with DAPI (#8961).
- For best results, allow mountant to cure overnight at room temperature. For long-term storage, store slides flat at 4°C protected from light.



# Notes:

# PathScan® Sandwich ELISA (ELISA)

## Colorimetric

last updated: 8/21/13

**NOTE:** Refer to product-specific datasheets or product webpage for assay incubation temperature.

### A. Solutions and Reagents

**NOTE:** Prepare solutions with reverse osmosis deionized (RODI) or equivalent grade water.

- 20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L PBS: add 50 ml 10X PBS to 950 ml dH<sub>2</sub>O, mix.
- Bring all microwell strips to room temperature before use.
- Prepare 1X Wash Buffer by diluting 20X Wash Buffer (included in each PathScan® Sandwich ELISA Kit) in dH<sub>2</sub>O.
- 1X Cell Lysis Buffer:** 10X Cell Lysis Buffer (#9803): To prepare 10 ml of 1X Cell Lysis Buffer, add 1 ml of 10X Cell Lysis Buffer to 9 ml of dH<sub>2</sub>O, mix. PathScan® Sandwich ELISA Lysis Buffer (#7018) 1X: This buffer is ready to use as is. Both buffers can be stored at 4°C for short-term use (1–2 weeks). **Recommended:** Add 1 mM phenylmethylsulfonyl fluoride (PMSF) (#8553) immediately before use. **NOTE:** Refer to product-specific datasheet or webpage for lysis buffer recommendation.
- TMB Substrate:** (#7004)
- STOP Solution:** (#7002)

### B. Preparing Cell Lysates

#### For adherent cells

- Aspirate media when the culture reaches 80–90% confluence. Treat cells by adding fresh media containing regulator for desired time.
- Remove media and rinse cells once with ice-cold 1X PBS.
- Remove PBS and add 0.5 ml ice-cold 1X cell lysis buffer plus 1 mM PMSF to each plate (10 cm diameter) and incubate the plate on ice for 5 min.
- Scrape cells off the plate and transfer to an appropriate tube. Keep on ice.
- Sonicate lysates on ice.
- Microcentrifuge for 10 min (x14,000 rpm) at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Store at -80°C in single-use aliquots.

#### For suspension cells

- Remove media by low speed centrifugation (~1,200 rpm) when the culture reaches 0.5–1.0 x 10<sup>6</sup> viable cells/ml. Treat cells by adding fresh media containing regulator for desired time.
- Collect cells by low speed centrifugation (~1,200 rpm) and wash once with 5–10 ml ice-cold 1X PBS.
- Cells harvested from 50 ml of growth media can be lysed in 2.0 ml of 1X cell lysis buffer plus 1 mM PMSF.
- Sonicate lysates on ice.
- Microcentrifuge for 10 min (x14,000 rpm) at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Store at -80°C in single-use aliquots.

### C. Test Procedure

- After the microwell strips have reached room temperature, break off the required number of microwells. Place the microwells in the strip holder. Unused microwells must be resealed in the storage bag and stored at 4°C immediately.
- Cell lysates can be undiluted or diluted with sample diluent (supplied in each PathScan® Sandwich ELISA Kit, blue color). Individual datasheets or product webpage for each kit provide information regarding an appropriate dilution factor for lysates and kit assay results.
- Add 100 µl of each undiluted or diluted cell lysate to the appropriate well. Seal with tape and press firmly onto top of microwells. Incubate the plate for 2 hr at 37°C. Alternatively, the plate can be incubated overnight at 4°C.
- Gently remove the tape and wash wells:**
  - Discard plate contents into a receptacle.
  - Wash 4 times with 1X wash buffer, 200 µl each time per well.
  - For each wash, strike plates on fresh paper towels hard enough to remove the residual solution in each well, but do not allow wells to completely dry at any time.
  - Clean the underside of all wells with a lint-free tissue.
- Add 100 µl of detection antibody (green color) to each well. Seal with tape and incubate the plate at 37°C for 1 hr.
- Repeat wash procedure (Section C, Step 4).
- Add 100 µl of HRP-linked secondary antibody (red color) to each well. Seal with tape and incubate the plate for 30 min at 37°C.
- Repeat wash procedure (Section C, Step 4).
- Add 100 µl of TMB substrate to each well. Seal with tape and incubate the plate for 10 min at 37°C or 30 min at 25°C.
- Add 100 µl of STOP solution to each well. Shake gently for a few seconds.

**NOTE:** Initial color of positive reaction is blue, which changes to yellow upon addition of STOP solution.

#### 11. Read results

- Visual Determination:** Read within 30 min after adding STOP solution.
- Spectrophotometric Determination:** Wipe underside of wells with a lint-free tissue. Read absorbance at 450 nm within 30 min after adding STOP solution.



# PathScan® Sandwich ELISA (ELISA)

## Chemiluminescent

last updated: 8/21/13

**NOTE:** Refer to product-specific datasheets or product webpage for assay incubation temperature. This chemiluminescent ELISA is offered in low volume microplate. Samples and reagents only require 50 µl per microwell.

### A. Solutions and Reagents

**NOTE:** Prepare solutions with reverse osmosis deionized (RODI) or equivalent grade water.

- 20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L 1X PBS: add 50 ml 10X PBS to 950 ml dH<sub>2</sub>O, mix.
- Bring all microwell strips to room temperature before use.
- Prepare 1X wash buffer by diluting 20X Wash Buffer (included in each PathScan® Sandwich ELISA Kit) in dH<sub>2</sub>O.
- 1X Cell Lysis Buffer:** 10X Cell Lysis Buffer (#9803): To prepare 10 ml of 1X Cell Lysis Buffer, add 1 ml of 10X Cell Lysis Buffer to 9 ml of dH<sub>2</sub>O, mix. PathScan® Sandwich ELISA Lysis Buffer (#7018) 1X: This buffer is ready to use as is. Both buffers can be stored at 4°C for short-term use (1–2 weeks). **Recommended:** Add 1 mM phenylmethylsulfonyl fluoride (PMSF) (#8553) immediately before use. **NOTE:** Refer to product-specific datasheet or webpage for lysis buffer recommendation.
- 20X LumiGLO® Reagent and 20X Peroxide:** (#7003)

### B. Preparing Cell Lysates

#### For adherent cells

- Aspirate media when the culture reaches 80–90% confluence. Treat cells by adding fresh media containing regulator for desired time.
- Remove media and rinse cells once with ice-cold 1X PBS.
- Remove PBS and add 0.5 ml ice-cold 1X Cell Lysis Buffer plus 1 mM PMSF to each plate (10 cm diameter) and incubate the plate on ice for 5 min.
- Scrape cells off the plate and transfer to an appropriate tube. Keep on ice.
- Sonicate lysates on ice.
- Microcentrifuge for 10 min (x14,000 rpm) at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Store at -80°C in single-use aliquots.

#### For suspension cells

- Remove media by low speed centrifugation (~1,200 rpm) when the culture reaches 0.5–1.0 x 10<sup>6</sup> viable cells/ml. Treat cells by adding fresh media containing regulator for desired time.
- Collect cells by low speed centrifugation (~1,200 rpm) and wash once with 5–10 ml ice-cold 1X PBS.
- Cells harvested from 50 ml of growth medium can be lysed in 2.0 ml of 1X cell lysis buffer plus 1 mM PMSF.
- Sonicate lysates on ice.
- Microcentrifuge for 10 min (x14,000 rpm) at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Store at -80°C in single-use aliquots.

### C. Test Procedure

- After the microwell strips have reached room temperature, break off the required number of microwells. Place the microwells in the strip holder. Unused microwells must be resealed in the storage bag and stored at 4°C immediately.
- Cell lysates can be used undiluted or diluted with sample diluent (supplied in each PathScan® Sandwich ELISA Kit, blue color). Individual datasheets or product webpage for each kit provide information regarding an appropriate dilution factor for lysates and kit assay results.
- Add 50 µl of each undiluted or diluted cell lysate to the appropriate well. Seal with tape and press firmly onto top of microwells. Incubate the plate for 2 hr at room temperature. Alternatively, the plate can be incubated overnight at 4°C.
- Gently remove the tape and wash wells:**
  - Discard plate contents into a receptacle.
  - Wash 4 times with 1X Wash Buffer, 150 µl each time per well.
  - For each wash, strike plates on fresh paper towels hard enough to remove the residual solution in each well, but do not allow wells to dry completely at any time.
  - Clean the underside of all wells with a lint-free tissue.
- Add 50 µl of detection antibody (green color) to each well. Seal with tape and incubate the plate at room temperature for 1 hr.
- Repeat wash procedure (Section C, Step 4).
- Add 50 µl of HRP-linked secondary antibody (red color) to each well. Seal with tape and incubate the plate at room temperature for 30 min.
- Repeat wash procedure (Section C, Step 4).
- Prepare detection reagent working solution by mixing equal parts 2X LumiGLO® Reagent and 2X Peroxide.
- Add 50 µl of the detection reagent working solution to each well.
- Use a plate-based luminometer set at 425 nm to measure Relative Light Units (RLU) within 1–10 min following addition of the substrate.
  - Optimal signal intensity is achieved when read within 10 min.



# PathScan® Sandwich ELISA (ELISA)

## Antibody Pair

last updated: 8/21/13

### A. Solutions and Reagents

**NOTE:** Prepare solutions with reverse osmosis deionized (RODI) or equivalent grade water.

- 20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L 1X PBS: add 50 ml 20X PBS to 950 ml dH<sub>2</sub>O, mix.
- Wash Buffer:** 1X PBS/0.05% Tween® 20, (20X PBST #9809)
- Blocking Buffer:** 1X PBS/0.05% Tween® 20, 1% BSA
- 1X Cell Lysis Buffer:** 10X Cell Lysis Buffer (#9803): To prepare 10 ml of 1X Cell Lysis Buffer, add 1 ml of 10X Cell Lysis Buffer to 9 ml of dH<sub>2</sub>O, mix. PathScan® Sandwich ELISA Lysis Buffer (#7018) 1X: This buffer is ready to use as is. Both buffers can be stored at 4°C for short-term use (1–2 weeks). **Recommended:** Add 1 mM phenylmethylsulfonyl fluoride (PMSF) (#8553) immediately before use. **NOTE:** Refer to product-specific datasheet or webpage for lysis buffer recommendation.
- Bovine Serum Albumin (BSA):** (#9998)
- TMB Substrate:** (#7004)
- STOP Solution:** (#7002)

**NOTE:** Reagents should be made fresh daily.

### B. Preparing Cell Lysates

#### For adherent cells

- Aspirate media when the culture reaches 80–90% confluence. Treat cells by adding fresh media containing regulator for desired time.
- Remove media and rinse cells once with ice-cold 1X PBS.
- Remove PBS and add 0.5 ml ice-cold 1X Cell Lysis Buffer plus 1 mM PMSF to each plate (10 cm diameter) and incubate the plate on ice for 5 min.
- Scrape cells off the plate and transfer to an appropriate tube. Keep on ice.
- Sonicate lysates on ice.
- Microcentrifuge for 10 min (x14,000 rpm) at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Store at -80°C in single-use aliquots.

#### For suspension cells

- Remove media by low speed centrifugation (~1,200 rpm) when the culture reaches 0.5–1.0 x 10<sup>6</sup> viable cells/ml. Treat cells by adding fresh media containing regulator for desired time.
- Collect cells by low speed centrifugation (~1,200 rpm) and wash once with 5–10 ml ice-cold 1X PBS.
- Cells harvested from 50 ml of growth media can be lysed in 2.0 ml of 1X cell lysis buffer plus 1 mM PMSF.
- Sonicate lysates on ice.
- Microcentrifuge for 10 min (x14,000 rpm) at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Store at -80°C in single-use aliquots.

### C. Coating Procedure

- Rinse microplate with 200 µl of dH<sub>2</sub>O, discard liquid. Blot on paper towel to make sure wells are dry.
- Dilute capture antibody 1:100 in 1X PBS. For a single 96 well plate, add 100 µl of capture antibody stock to 9.9 ml 1X PBS. Mix well and add 100 µl/well. Cover plate and incubate overnight at 4°C (17–20 hr).
- After overnight coating, gently uncover plate and wash wells:**
  - Discard plate contents into a receptacle.
  - Wash four times with wash buffer, 200 µl each time per well. For each wash, strike plates on fresh paper towels hard enough to remove the residual solution in each well, but do not allow wells to completely dry at any time.
  - Clean the underside of all wells with a lint-free tissue.
- Block plates. Add 150 µl of blocking buffer/well, cover plate, and incubate at 37°C for 2 hr.
- After blocking, wash plate (Section C, Step 3). Plate is ready to use.

### D. Test Procedure

- Lysates can be used undiluted or diluted in blocking buffer. 100 µl of lysate is added per well. Cover plate and incubate at 37°C for 2 hr.
- Wash plate (Section C, Step 3).
- Dilute detection antibody 1:100 in blocking buffer. For a single 96 well plate, add 100 µl of detection antibody Stock to 9.9 ml of blocking buffer. Mix well and add 100 µl/well. Cover plate and incubate at 37°C for 1 hr.
- Wash plate (Section C, Step 3).
- Secondary antibody, either streptavidin anti-mouse or anti-rabbit-HRP, is diluted 1:1000 in blocking buffer. For a single 96 well plate, add 10 µl of secondary antibody stock to 9.99 ml of blocking buffer. Mix well and add 100 µl/well. Cover and incubate at 37°C for 30 min.
- Wash plate (Section C, Step 3).
- Add 100 µl of TMB substrate per well. Cover and incubate at 37°C for 10 min.
- Add 100 µl of STOP solution per well. Shake gently for a few seconds.
  - Visual Determination:** Read within 30 min after adding STOP solution.
  - Spectrophotometric Determination:** Wipe underside of wells with a lint-free tissue. Read absorbance at 450 nm within 30 min after adding STOP solution.



# Notes:

# Chromatin Immunoprecipitation (ChIP)

last updated: 8/21/13

## Enzymatic (pg 1 of 3)

### Solutions and Reagents Included:

**Reagents Included in SimpleChIP® and SimpleChIP® Plus Enzymatic Chromatin IP Kits #9002, #9003, #9004, or #9005:** Glycine Solution (10X) | Buffer A (4X) | Buffer B (4X) | ChIP Buffer (10X) | ChIP Elution Buffer (2X) | 5 M NaCl | 0.5 M EDTA | 1 M DTT | DNA Binding Reagent A | DNA Wash Reagent B | DNA Elution Reagent C | DNA Spin Columns | Protease Inhibitor Cocktail (200X) | RNase A (10 mg/ml) | Micrococcal Nuclease (2000 gel units/μl) | Proteinase K (20 mg/ml) | SimpleChIP® Human RPL30 Exon 3 Primers #7014 | SimpleChIP® Mouse RPL30 Intron 2 Primers #7015 | Histone H3 (D2B12) XP® Rabbit mAb (ChIP Formulated) #4620 | Normal Rabbit IgG #2729 | ChIP-Grade Protein G Magnetic Beads #9006 or ChIP-Grade Protein G Agarose Beads #9007

### Reagents Not Included:

Formaldehyde (37%) | Ethanol (96-100%) | Isopropanol | 1X PBS (20X PBS #9808) | Nuclease-free water | Taq DNA polymerase | dNTP Mix | **For Kits #9002 and #9003 only:** PMSF (#8553) (0.1 M stock) | **For Kits #9003 and #9005 only:** 6-Tube Magnetic Separation Rack #7017

### I. Tissue Cross-linking and Sample Preparation

**IMPORTANT:** Section I exclusive to use of SimpleChIP® Plus Kits (#9004 & #9005)

When harvesting tissue, remove unwanted material such as fat and necrotic material from the sample. Tissue can then be processed and cross-linked immediately, or frozen on dry ice for processing later. For optimal chromatin yield and ChIP results, use 25 mg of tissue for each IP to be performed. The chromatin yield does vary between tissue types and some tissues may require more than 25 mg for each IP. Please see Troubleshooting Guide, Section A for more information regarding the expected chromatin yield for different types of tissue. One additional chromatin sample should be processed for Analysis of Chromatin Digestion and Concentration (Section IV).

#### Before starting:

- Remove and warm 200X Protease Inhibitor Cocktail (PIC) and 10X glycine solution. Make sure PIC is completely thawed.
- Prepare 3 ml of 1X Phosphate Buffered Saline (PBS) + 15 μl 200X PIC per 25 mg of tissue to be processed and place on ice.
- Prepare 45 μl of 37% formaldehyde per 25 mg of tissue to be processed and keep at room temperature. Use fresh formaldehyde that is not past the manufacturer's expiration date.

### A. Cross-linking

1. Weigh the fresh or frozen tissue sample. Use 25 mg of tissue for each IP to be performed.
2. Place tissue sample in a 60 mm or 100 mm dish and finely mince using a clean scalpel or razor blade. Keep dish on ice. It is important to keep the tissue cold to avoid protein degradation.
3. Transfer minced tissue to a 15 ml conical tube.

4. Add 1 ml of 1X PBS + PIC per 25 mg tissue to the conical tube.
5. To crosslink proteins to DNA, add 45 μl of 37% formaldehyde per 1 ml of 1X PBS + PIC and rock at room temp for 20 min. Final formaldehyde concentration is 1.5%.
6. Stop cross-linking by adding 100 μl of 10X glycine per 1 ml of 1X PBS + PIC and mix for 5 min at room temperature.
7. Centrifuge tissue at 1,500 rpm in a bench top centrifuge for 5 min at 4°C.
8. Remove supernatant and wash with 1 ml 1X PBS + PIC per 25 mg tissue.
9. Repeat centrifugation (Section A, Step 7).
10. Remove supernatant and resuspend tissue in 1 ml 1X PBS + PIC per 25 mg tissue and store on ice. Disaggregate tissue into single-cell suspension using a Medimachine (Section B) or Dounce homogenizer (Section C).

### B. Tissue Disaggregation Using Medimachine from BD™ Biosciences (part #340587)

1. Cut off the end of a 1 ml pipette tip to enlarge the opening for transfer of tissue chunks.
2. Transfer 1 ml of tissue resuspended in 1X PBS + PIC into the top chamber of a 50 mm medicone (Part #340592).
3. Grind tissue for 2 min according to manufacturer's instructions.
4. Collect cell suspension from the bottom chamber of the medicone using a 1 ml syringe and 18-gauge blunt needle. Transfer cell suspension to a 15 ml conical tube and place on ice.
5. Repeat steps 2–4 until all the tissue is processed into a homogenous suspension.
6. If more grinding is necessary, add more 1X PBS + PIC to tissue. Repeat steps 2 to 5 until all tissue is ground into a homogeneous suspension.
7. Check for single-cell suspension by microscope (optional).
8. Centrifuge cells at 1,500 rpm in a bench top centrifuge for 5 min at 4°C.
9. Remove supernatant from cells and immediately continue with Nuclei Preparation and Chromatin Digestion (Section III).

### C. Tissue Disaggregation Using a Dounce Homogenizer

1. Transfer tissue resuspended in 1X PBS + PIC to a Dounce homogenizer.
2. Disaggregate tissue pieces with 20–25 strokes. Check for single-cell suspension by microscope (optional).
3. Transfer cell suspension to a 15 ml conical tube and centrifuge at 1,500 rpm in a bench top centrifuge for 5 min at 4°C.
4. Remove supernatant from cells and immediately continue with Nuclei Preparation and Chromatin Digestion (Section III).

### II. Cell Culture Cross-linking and Sample Preparation

For optimal ChIP results, use approximately  $4 \times 10^6$  cells for each IP to be performed. For HeLa cells, this is equivalent to half of a 15 cm culture dish containing cells that are 90% confluent in 20 ml of growth medium. One additional sample should be processed for Analysis of Chromatin Digestion and Concentration (Section IV). Include one extra dish of cells in experiment to be used for determination of cell number using a hemocytometer.

#### Before starting:

- Remove and warm 200X Protease Inhibitor Cocktail (PIC) and 10X Glycine Solution. Make sure PIC is completely thawed.
- Prepare 2 ml of Phosphate Buffered Saline (PBS) + 10 μl 200X PIC per 15 cm dish to be processed and place on ice.
- Prepare 40 ml of 1X PBS per 15 cm dish to be processed and place on ice.
- Prepare 540 μl of 37% formaldehyde per 15 cm dish of cells to be processed and keep at room temperature. Use fresh formaldehyde that is not past the manufacturer's expiration date.

1. To crosslink proteins to DNA, add 540 μl of 37% formaldehyde to each 15 cm culture dish containing 20 ml medium. Swirl briefly to mix and incubate 10 min at room temperature. Final formaldehyde concentration is 1%. Addition of formaldehyde may result in a color change of the medium.
2. Add 2 ml of 10X glycine solution to each 15 cm dish containing 20 ml medium, swirl briefly to mix, and incubate 5 min at room temperature. Addition of glycine may result in a color change of the medium.
3. For suspension cells, transfer cells to a 50 ml conical tube, centrifuge at 1,500 rpm in a bench top centrifuge for 5 min at 4°C and wash pellet two times with 20 ml ice-cold 1X PBS. Remove supernatant and immediately continue with Nuclei Preparation and Chromatin Digestion (Section III).
4. For adherent cells, remove media and wash cells two times with 20 ml ice-cold 1X PBS, completely removing wash from culture dish each time.
5. Add 2 ml ice-cold 1X PBS + PIC to each 15 cm dish. Scrape cells into cold buffer. Combine cells from all culture dishes into one 15 ml conical tube.
6. Centrifuge cells at 1,500 rpm in a bench top centrifuge for 5 min at 4°C. Remove supernatant and immediately continue with Nuclei Preparation and Chromatin Digestion (Section III).

### III. Nuclei Preparation and Chromatin Digestion

One IP preparation is defined as 25 mg of disaggregated tissue or  $4 \times 10^6$  tissue culture cells.

#### Before starting:

- Remove and warm 200X Protease Inhibitor Cocktail (PIC) and 1 M DTT. Make sure both are completely thawed and DTT crystals are completely in solution.



# Chromatin Immunoprecipitation (ChIP)

last updated: 8/21/13

## Enzymatic (pg 2 of 3)

- Remove and warm 10X ChIP buffer and ensure SDS is completely in solution.
  - Prepare 1 ml 1X buffer A (250 µl 4X buffer A + 750 µl water) + 0.5 µl 1 M DTT + 5 µl 200X PIC per IP prep and place on ice.
  - Prepare 1.1 ml 1X Buffer B (275 µl 4X buffer B + 825 µl water) + 0.55 µl 1 M DTT per IP prep and place on ice.
  - Prepare 100 µl 1X ChIP buffer (10 µl 10X ChIP Buffer + 90 µl water) + 0.5 µl 200X PIC per IP prep and place on ice.
1. Resuspend cells in 1 ml ice-cold buffer A + DTT + PIC per IP prep. Incubate on ice for 10 min. Mix by inverting tube every 3 min.
  2. Pellet nuclei by centrifugation at 3,000 rpm in a bench top centrifuge for 5 min at 4°C. Remove supernatant and resuspend pellet in 1 ml ice-cold buffer B + DTT per IP prep. Repeat centrifugation, remove supernatant, and resuspend pellet in 100 µl Buffer B + DTT per IP prep. Transfer sample to a 1.5 ml microcentrifuge tube, up to 1 ml total per tube.
  3. Add 0.5 µl of micrococcal nuclease per IP prep, mix by inverting tube several times and incubate for 20 min at 37°C with frequent mixing to digest DNA to length of approximately 150–900 bp. Mix by inversion every 3–5 min. The amount of micrococcal nuclease required to digest DNA to the optimal length may need to be determined empirically for individual tissues and cell lines (see Troubleshooting Guide, Section B). HeLa nuclei digested with 0.5 µl micrococcal nuclease per 4 x 10<sup>6</sup> cells and mouse liver tissue digested with 0.5 µl micrococcal nuclease per 25 mg of tissue gave the appropriate length DNA fragments.
  4. Stop digest by adding 10 µl of 0.5 M EDTA per IP prep and placing tube on ice.
  5. Pellet nuclei by centrifugation at 13,000 rpm in a microcentrifuge for 1 min at 4°C and remove supernatant.
  6. Resuspend nuclear pellet in 100 µl of 1X ChIP buffer + PIC per IP prep and incubate on ice for 10 min.
  7. Sonicate up to 500 µl of lysate per 1.5 ml microcentrifuge tube with several pulses to break nuclear membrane. Incubate samples for 30 sec on wet ice between pulses. Optimal conditions required for complete lysis of nuclei can be determined by observing nuclei on a light microscope before and after sonication.

HeLa nuclei were completely lysed after 3 sets of 20-sec pulses using a VirTis Virsonic 100 Ultrasonic Homogenizer/Sonicator at setting 6 with a 1/8-inch probe. Alternatively, nuclei can be lysed by homogenizing the lysate 20 times in a Dounce homogenizer; however, lysis may not be as complete.
  8. Clarify lysates by centrifugation at 10,000 rpm in a microcentrifuge for 10 min at 4°C.
  9. Transfer supernatant to a new tube. This is the cross-linked chromatin preparation, which should be stored at -80°C until further use. Remove 50 µl of the chromatin preparation for Analysis of Chromatin Digestion and Concentration (Section IV).

### IV. Analysis of Chromatin Digestion and Concentration

#### (Recommended Step)

1. To the 50 µl chromatin sample (Step 9 in Section III), add 100 µl nuclease-free water, 6 µl 5 M NaCl, and 2 µl RNase A. Vortex to mix and incubate samples at 37°C for 30 min.
2. To each RNase A-digested sample, add 2 µl Proteinase K. Vortex to mix and incubate samples at 65°C for 2 hr.
3. Purify DNA from samples using DNA purification spin columns as described in Section VII.
4. After purification of DNA, remove a 10 µl sample and determine DNA fragment size by electrophoresis on a 1% agarose gel with a 100 bp DNA marker. DNA should be digested to a length of approximately 150–900 bp (1–6 nucleosomes).
5. To determine DNA concentration, transfer 2 µl of purified DNA to 98 µl nuclease-free water to give a 50-fold dilution and read the OD<sub>260</sub>. The concentration of DNA in µg/ml is OD<sub>260</sub> x 2,500. DNA concentration should ideally be between 50 and 200 µg/ml.

**NOTE:** For optimal ChIP results, it is highly critical that the chromatin is of appropriate size and concentration. Over-digestion of chromatin may diminish signal in the PCR quantification. Under-digestion of chromatin may lead to increased background signal and lower resolution. Adding too little chromatin to the IP may result in diminished signal in the PCR quantification. A protocol for optimization of chromatin digestion can be found in the Troubleshooting Guide.

### V. Chromatin IP

For optimal ChIP results, use approximately 5 to 10 µg of digested, cross-linked chromatin (as determined in Section IV) per IP. This should be roughly equivalent to a single 100 µl IP prep from 25 mg of disaggregated tissue or 4 x 10<sup>6</sup> tissue culture cells. Typically, 100 µl of digested chromatin is diluted into 400 µl 1X ChIP Buffer prior to the addition of antibodies. However, if more than 100 µl of chromatin is required per IP, the cross-linked chromatin preparation does not need to be diluted as described below. Antibodies can be added directly to the undiluted chromatin preparation for IP of chromatin complexes.

#### Before starting:

- Remove and warm 200X Protease Inhibitor Cocktail (PIC). Make sure PIC is completely thawed.
- Remove and warm 10X ChIP Buffer and ensure SDS is completely in solution.
- Thaw digested chromatin preparation (Section III, Step 9) and place on ice.
- Prepare low salt wash: 3 ml 1X ChIP buffer (300 µl 10X ChIP buffer + 2.7 ml H<sub>2</sub>O) per IP prep. Store at room temperature until use.
- Prepare high salt wash: 1 ml 1X ChIP buffer (100 µl 10X ChIP buffer + 900 µl H<sub>2</sub>O) + 70 µl 5M NaCl per IP. Store at room temperature until use.

1. In one tube, prepare enough 1X ChIP buffer for the dilution of digested chromatin into the desired number of IPs: 400 µl of 1X ChIP buffer (40 µl of 10X ChIP buffer + 360 µl H<sub>2</sub>O) + 2 µl 200X PIC per IP. When determining the number of IPs, remember to include the positive control Histone H3 (D2B12) XP® Rabbit mAb and negative control Normal Rabbit IgG antibody samples. Place mix on ice.
2. To the prepared 1X ChIP buffer, add the equivalent of 100 µl (5–10 µg of chromatin) of the digested, cross-linked chromatin preparation (Section III, Step 9) per IP. For example, for 10 IPs, prepare a tube containing 4 ml 1X ChIP Buffer (400 µl 10X ChIP buffer + 3.6 ml water) + 20 µl 200X PIC + 1 ml digested chromatin preparation.
3. Remove a 10 µl sample of the diluted chromatin and transfer to a microfuge tube. This is your 2% input sample, which can be stored at -20°C until further use (Step 1 in Section VI).
4. For each IP, transfer 500 µl of the diluted chromatin to a 1.5 ml microcentrifuge tube and add the immunoprecipitating antibody. The amount of antibody required per IP varies and should be determined by the user. For the positive control Histone H3 (D2B12) XP® Rabbit mAb add 10 µl to the IP sample. For the negative control, Normal Rabbit IgG, add 1 µl (1 µg) to 2 µl (2 µg) to the IP sample. Incubate IP samples 4 hr to overnight at 4°C with rotation.
5. a. Resuspend ChIP-Grade Protein G Agarose Beads by gently vortexing. Immediately add 30 µl of Protein G Agarose Beads to each IP reaction and incubate for 2 hr at 4°C with rotation.  
b. Resuspend ChIP-Grade Protein G Magnetic Beads by gently vortexing. Immediately add 30 µl of Protein G Magnetic Beads to each IP reaction and incubate for 2 hr at 4°C with rotation.
6. a. Pellet Protein G Agarose Beads in each IP by brief 1 min centrifugation at 6,000 rpm in a microcentrifuge and remove supernatant.  
b. Pellet Protein G Magnetic Beads in each IP by placing the tubes in a Magnetic Separation Rack. Wait 1–2 min for solution to clear and then carefully remove supernatant.
7. Wash Protein G Beads by adding 1 ml of low salt wash to the beads and incubate at 4°C for 5 min with rotation. Repeat Steps 6 and 7 two additional times for a total of 3 low salt washes.
8. Add 1 ml of high salt wash to the beads and incubate at 4°C for 5 min with rotation.
9. a. Pellet Protein G Agarose Beads in each IP by brief 1 min centrifugation at 6,000 rpm in a microcentrifuge. Remove supernatant and immediately proceed to Section VI.  
b. Pellet Protein G Magnetic Beads in each IP by placing the tubes in a Magnetic Separation Rack. Wait 1–2 min for solution to clear and then carefully remove supernatant and proceed to Section VI.



# Chromatin Immunoprecipitation (ChIP)

last updated: 8/21/13

## Enzymatic (pg 3 of 3)

### VI. Elution of Chromatin from Antibody/Protein G Agarose Beads and Reversal of Cross-links

#### Before starting:

- Remove and warm 2X ChIP elution buffer in a 37°C water bath and ensure SDS is in solution.
  - Set a water bath or thermomixer to 65°C.
  - Prepare 150 µl 1X ChIP elution buffer (75 µl 2X ChIP elution buffer + 75 µl water) for each IP and the 2% input sample.
1. Add 150 µl of the 1X ChIP elution buffer to the 2% input sample tube and set aside at room temperature until Step 6.
  2. Add 150 µl 1X ChIP elution buffer to each IP sample.
  3. Elute chromatin from the antibody/Protein G Beads for 30 min at 65°C with gentle vortexing (1,200 rpm). A thermomixer works best for this step. Alternatively, elutions can be performed at room temperature with rotation, but may not be as complete.
  4. **a.** Pellet Protein G Agarose Beads by brief 1 min centrifugation at 6,000 rpm in a microcentrifuge.  
**b.** Pellet Protein G Magnetic Beads by placing the tubes in a Magnetic Separation Rack and wait 1 to 2 min for solution to clear.
  5. Carefully transfer eluted chromatin supernatant to a new tube.
  6. To all tubes, including the 2% input sample from Step 1, reverse cross-links by adding 6 µl 5 M NaCl and 2 µl Proteinase K, and incubate 2 hr at 65°C. This incubation can be extended overnight.
  7. Immediately proceed to Section VII. Alternatively, samples can be stored at -20°C. However, to avoid formation of a precipitate, be sure to warm samples to room temperature before adding DNA binding reagent A (Section VII, Step 1).

### VII. DNA Purification Using Spin Columns

#### Before starting:

- Add 12 ml of isopropanol to DNA binding reagent A and 24 ml of ethanol (96–100%) to DNA wash reagent B before use. These steps only have to be performed once prior to the first set of DNA purifications.
  - Remove one DNA purification spin column and collection tube for each DNA sample (Section VI).
1. Add 600 µl DNA binding reagent A to each DNA sample and vortex briefly. 4 volumes of DNA binding reagent A should be used for every 1 volume of sample.
  2. Transfer 375 µl of each sample from Step 1 to a DNA purification spin column in collection tube.
  3. Centrifuge at 14,000 rpm in a microcentrifuge for 30 sec.
  4. Remove spin column from the collection tube and discard the liquid. Replace spin column in the collection tube.

5. Transfer remaining 375 µl of each sample from Step 1 to the spin column in collection tube. Repeat Steps 3 and 4.
6. Add 700 µl of DNA wash reagent B to the spin column in collection tube.
7. Centrifuge at 14,000 rpm in a microcentrifuge for 30 sec.
8. Remove spin column from the collection tube and discard the liquid. Replace spin column in the collection tube.
9. Centrifuge at 14,000 rpm in a microcentrifuge for 30 sec.
10. Discard collection tube and liquid. Retain spin column.
11. Add 50 µl of DNA elution reagent C to each spin column and place into a clean 1.5 ml microcentrifuge tube.
12. Centrifuge at 14,000 rpm in a microcentrifuge for 30 sec to elute DNA.
13. Remove and discard DNA purification spin column. Eluate is now purified DNA. Samples can be stored at -20°C.

### VIII. Quantification of DNA by PCR

#### Recommendations:

- Use Filter-tip pipette tips to minimize risk of contamination.
- The control primers included in the kit are specific for the human or mouse RPL30 gene and can be used for either standard PCR or quantitative real-time PCR. If the user is performing ChIPs from another species, it is recommended that the user design the appropriate specific primers to DNA and determine the optimal PCR conditions.
- A Hot-Start Taq polymerase is recommended to minimize the risk of non-specific PCR products.
- PCR primer selection is critical. Primers should be designed with close adherence to the following criteria:  
**Primer length:** 24 nucleotides  
**Optimum Tm:** 60°C  
**Optimum GC:** 50%  
**Amplicon size:** 150–200 bp (for standard PCR) 80–160 bp (for real-time quantitative PCR)

#### Standard PCR Method:

1. Label the appropriate number of 0.2 ml PCR tubes for the number of samples to be analyzed. These should include the 2% input sample, the positive control histone H3 sample, the negative control normal rabbit IgG sample, and a tube with no DNA to control for DNA contamination.
2. Add 2 µl of the appropriate DNA sample to each tube.
3. Prepare a master reaction mix as described below, making sure to add enough reagent for two extra tubes to account for loss of volume. Add 18 µl of master mix to each reaction tube.

**Reagent:** Volume for 1 PCR Reaction (18 µl) | Nuclease-free H<sub>2</sub>O: 12.5 µl | 10X PCR Buffer: 2.0 µl | 4 mM dNTP Mix: 1.0 µl | 5 µM RPL30 Primers: 2.0 µl | Taq DNA Polymerase: 0.5 µl

#### 4. Start the following PCR reaction program:

- a. Initial denaturation 95°C 5 min
- b. Denature 95°C 30 sec
- c. Anneal 62°C 30 sec
- d. Extension 72°C 30 sec
- e. Repeat Steps b–d for a total of 34 cycles
- f. Final Extension 72°C 5 min

5. Remove 10 µl of each PCR product for analysis by 2% agarose gel or 10% polyacrylamide gel electrophoresis with a 100 bp DNA marker. The expected size of the PCR product is 161 bp for human RPL30 and 159 bp for mouse RPL30.

#### Real-Time Quantitative PCR Method:

1. Label the appropriate number of PCR tubes or PCR plates compatible with the model of PCR machine to be used. PCR reactions should include the positive control histone H3 sample, the negative control normal rabbit IgG sample, a tube with no DNA to control for contamination, and a serial dilution of the 2% input chromatin DNA (undiluted, 1:5, 1:25, 1:125) to create a standard curve and determine the efficiency of amplification.
2. Add 2 µl of the appropriate DNA sample to each tube or well of the PCR plate.
3. Prepare a master reaction mix as described below. Add enough reagents for two extra reactions to account for loss of volume. Add 18 µl of reaction mix to each PCR reaction tube or well.  
**Reagent:** Volume for 1 PCR Reaction (18 µl) | Nuclease-free H<sub>2</sub>O: 6 µl | 5 µM RPL30 Primers: 2 µl | 2X SYBR®-Green Reaction Mix: 10 µl
4. Start the following PCR reaction program:
  - a. Initial denaturation 95°C, 3 min
  - b. Denature 95°C, 15 sec
  - c. Anneal and extension: 60°C, 60 sec
  - d. Repeat steps b and c for a total of 40 cycles.
5. Analyze quantitative PCR results using the software provided with the real-time PCR machine. Alternatively, one can calculate the IP efficiency manually using the Percent Input Method and the equation shown below. With this method, signals obtained from each IP are expressed as a percent of the total input chromatin.

$$\text{Percent Input} = 2\% \times 2^{(C[T] - 2\% \text{ Input Sample} - C[T] \text{ IP Sample})}$$

$C[T] = C_t$  = Threshold cycle of PCR reaction



Cell Signaling  
TECHNOLOGY®

# Notes:

# Chromatin Immunoprecipitation (ChIP)

last updated: 8/21/13

## Troubleshooting Guide (pg 1 of 2)

### A. Expected Chromatin Yield

When harvesting cross-linked chromatin from tissue samples, the yield of chromatin can vary significantly between tissue types. The table below provides a range for the expected yield of chromatin from 25 mg of tissue compared to  $4 \times 10^6$  HeLa cells, and the expected DNA concentration, as determined in Section IV of the protocol. For each tissue type, disaggregation using a BD™ Medimachine system (BD Biosciences) or a Dounce homogenizer yielded similar amounts of chromatin. However, chromatin processed from tissues disaggregated using the Medimachine typically gave higher IP efficiencies than chromatin processed from tissues disaggregated using a Dounce homogenizer. A Dounce homogenizer is strongly recommended for disaggregation of brain tissue, as the Medimachine does not adequately disaggregate brain tissue into a single-cell suspension. For optimal ChIP results, we recommend using 5 to 10 µg of digested, cross-linked chromatin per IP; therefore, some tissues may require harvesting more than 25 mg per each IP.

| Tissue/Cell   | Total Chromatin Yield              | Expected DNA Concentration |
|---------------|------------------------------------|----------------------------|
| <b>Spleen</b> | 20–30 µg per 25 mg tissue          | 200–300 µg/ml              |
| <b>Liver</b>  | 10–15 µg per 25 mg tissue          | 100–150 µg/ml              |
| <b>Kidney</b> | 8–10 µg per 25 mg tissue           | 80–100 µg/ml               |
| <b>Brain</b>  | 2–5 µg per 25 mg tissue            | 20–50 µg/ml                |
| <b>Heart</b>  | 2–5 µg per 25 mg tissue            | 20–50 µg/ml                |
| <b>HeLa</b>   | 10–15 µg per $4 \times 10^6$ cells | 100–150 µg/ml              |

### B. Optimization of Chromatin Digestion

Optimal conditions for the digestion of cross-linked chromatin DNA to 150–900 bp in length is highly dependent on the ratio of micrococcal nuclease to the amount of tissue or number of cells used in the digest. Below is a protocol for determination of the optimal digestion conditions for a specific tissue or cell type.

1. Prepare cross-linked nuclei from 125 mg of tissue or  $2 \times 10^7$  cells (equivalent of 5 IP preps), as described in Protocol Sections I, II, and III. Stop after Step 2 of Protocol Section III and proceed as described below.
2. Transfer 100 µl of the nuclei preparation into 5 individual 1.5 ml microcentrifuge tubes and place on ice.
3. Add 3 µl micrococcal nuclease stock to 27 µl of 1X Buffer B + DTT (1:10 dilution of enzyme).

4. To each of the 5 tubes in Step 2, add 0 µl, 2.5 µl, 5 µl, 7.5 µl, or 10 µl of the diluted micrococcal nuclease, mix by inverting tube several times and incubate for 20 min at 37°C with frequent mixing.
5. Stop each digest by adding 10 µl of 0.5 M EDTA and placing tubes on ice.
6. Pellet nuclei by centrifugation at 13,000 rpm in a microcentrifuge for 1 min at 4°C and remove supernatant.
7. Resuspend nuclear pellet in 200 µl of 1X ChIP buffer + PIC. Incubate on ice for 10 min.
8. Sonicate lysate with several pulses to break nuclear membrane. Incubate samples for 30 sec on wet ice between pulses. Optimal conditions required for complete lysis of nuclei can be determined by observing nuclei on a light microscope before and after sonication. HeLa nuclei were completely lysed after 3 sets of 20 sec pulses using a VirTis Virsonic 100 Ultrasonic Homogenizer/Sonicator set at setting 6 with a 1/8-inch probe. Alternatively, nuclei can be lysed by homogenizing the lysate 20 times in a Dounce homogenizer; however, lysis may not be as complete.
9. Clarify lysates by centrifugation at 10,000 rpm in a microcentrifuge for 10 min at 4°C.
10. Transfer 50 µl of each of the sonicated lysates to new microfuge tubes.
11. To each 50 µl sample, add 100 µl nuclease-free water, 6 µl 5 M NaCl and 2 µl RNase A. Vortex to mix and incubate samples at 37°C for 30 min.
12. To each RNase A-digested sample, add 2 µl Proteinase K. Vortex to mix and incubate sample at 65°C for 2 hr.
13. Remove 20 µl of each sample and determine DNA fragment size by electrophoresis on a 1% agarose gel with a 100 bp DNA marker.
14. Observe which of the digestion conditions produces DNA in the desired range of 150–900 base pairs (1–6 nucleosomes). The volume of diluted micrococcal nuclease that produces the desired size of DNA fragments using this optimization protocol is equivalent to 10 times the volume of micrococcal nuclease stock that should be added to one IP preparation (25 mg of disaggregated tissue cells or  $4 \times 10^6$  tissue culture cells) to produce the desired size of DNA fragments. For example, if 5 µl of diluted micrococcal nuclease produces DNA fragments of 150–900 bp in this protocol, then 0.5 µl of stock micrococcal nuclease should be added to one IP preparation during the digestion of chromatin in Section III.
15. If results indicate that DNA is not in the desired size range, then repeat optimization protocol, adjusting the amount of micrococcal nuclease in each digest accordingly. Alternatively, the digestion time can be changed to increase or decrease the extent of DNA fragmentation.



# Chromatin Immunoprecipitation (ChIP)

last updated: 8/21/13

## Troubleshooting Guide (pg 2 of 2)

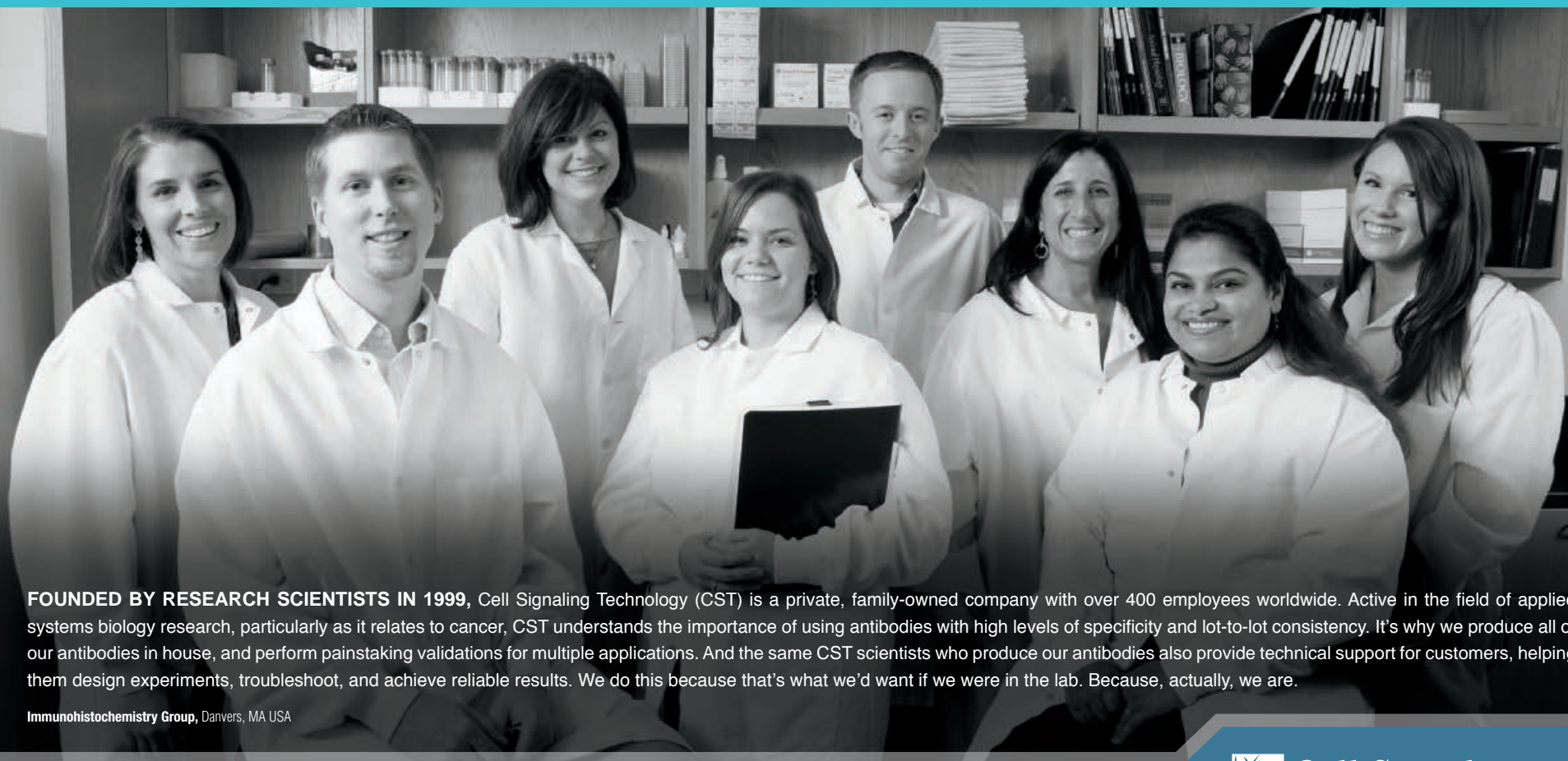
| Problem   | Possible Causes  | Recommendation  |
|---|--|---|
| <b>Concentration of the digested chromatin is too low (low chromatin yield).</b>  | Not enough tissue or cells were added to the chromatin digestion or cell nuclei were not completely lysed after digestion. | Add additional chromatin to each IP to give at least 5 µg/IP and continue with protocol.  |
|   |  | Weigh tissue or count a separate plate of cells prior to cross-linking to determine accurate cell number. Some tissues may require processing of more than 25 mg per IP. The amount of tissue can be increased to 50 mg per IP, while still maintaining efficient chromatin fragmentation and extraction.   |
|   |  | Increase the number of sonications following chromatin digestion. Visualize cell nuclei under microscope before and after sonication to confirm complete lysis of nuclei.   |
| <b>Chromatin is under-digested and fragments are too large (greater than 900 bp). Large chromatin fragments can lead to increased background and lower resolution.</b>  | Too many cells or not enough micrococcal nuclease was added to the chromatin digestion.                                    | Weigh tissue or count a separate plate of cells prior to cross-linking to determine accurate cell number. Add less tissue or cells, or more micrococcal nuclease to the chromatin digest. See Section B for optimization of chromatin digestion.  |
|   | Tissue or cells may have been over cross-linked. Cross-linking for longer than 10 min may inhibit digestion of chromatin.  | Perform a time course at a fixed formaldehyde concentration. Shorten the time of cross-linking to 10 min or less.   |
| <b>Chromatin is over-digested and fragments are too small (exclusively 150 bp mono-nucleosome length). Complete digestion of chromatin to mono-nucleosome length DNA may diminish signal during PCR quantification, especially for amplicons greater than 150 bp in length.</b> | Not enough cells or too much micrococcal nuclease added to the chromatin digestion.  | Weigh tissue or count a separate plate of cells prior to cross-linking to determine accurate cell number. Add more tissue or cells, or less micrococcal nuclease to the chromatin digest. See Section B of troubleshooting guide for optimization of chromatin digestion.   |
| <b>No product or very little product in the input PCR reactions.</b>  | Not enough DNA added to the PCR reaction or conditions are not optimal.  | Add more DNA to the PCR reaction or increase the number of amplification cycles.  |
|   | PCR amplified region may span nucleosome-free region.  | Optimize the PCR conditions for experimental primer set using purified DNA from cross-linked and digested chromatin. Design a different primer set and decrease length of amplicon to less than 150 bp (see primer design recommendations in Protocol Section VIII).  |
|   | Not enough chromatin added to the IP or chromatin is over-digested.  | For optimal ChIP results, add 5 to 10 µg chromatin per IP.  |
| <b>No product in the positive control histone H3-IP RPL30 PCR reaction.</b>   | Not enough chromatin or antibody added to the IP reaction or IP incubation time is too short.                              | Be sure to add 5 to 10 µg of chromatin and 10 µl of antibody to each IP reaction and incubate with antibody overnight and an additional 2 hr after adding Protein G beads.  |
|   | Incomplete elution of chromatin from Protein G beads.  | Elution of chromatin from Protein G beads is optimal at 65°C with frequent mixing to keep beads suspended in solution.  |
| <b>Quantity of product in the negative control Rabbit IgG-IP and positive control histone H3-IP PCR reactions is equivalent (high background signal).</b>   | Too much or not enough chromatin added to the IP reaction. Alternatively, too much antibody added to the IP reaction.      | For optimal ChIP results, add 5 to 10 µg of chromatin and 10 µl of histone H3 antibody to each IP reaction. Reduce the amount of normal rabbit IgG to 1 µl per IP.  |
|   | Too much DNA added to the PCR reaction or too many cycles of amplification.  | Add less DNA to the PCR reaction or decrease the number of PCR cycles. It is very important that the PCR products are analyzed within the linear amplification phase of PCR. Otherwise, the differences in quantities of starting DNA cannot be accurately measured. Alternatively, quantify immunoprecipitations using real-time quantitative PCR. |
| <b>No product in the Experimental Antibody-IP PCR reaction.</b>   | Not enough DNA added to the PCR reaction.  | Add more DNA to the PCR reaction or increase the number of amplification cycles.  |
|   | Not enough antibody added to the IP reaction.  | Typically a range of 1 to 5 µg of antibody are added to the IP reaction; however, the exact amount depends greatly on the individual antibody. Increase the amount of antibody added to the IP.   |
|   | Antibody does not work for ChIP.   | Find an alternate antibody source.  |





our mission...

To deliver the world's highest quality research, diagnostic, and therapeutic products that accelerate biological understanding and enable personalized medicine.



**FOUNDED BY RESEARCH SCIENTISTS IN 1999**, Cell Signaling Technology (CST) is a private, family-owned company with over 400 employees worldwide. Active in the field of applied systems biology research, particularly as it relates to cancer, CST understands the importance of using antibodies with high levels of specificity and lot-to-lot consistency. It's why we produce all of our antibodies in house, and perform painstaking validations for multiple applications. And the same CST scientists who produce our antibodies also provide technical support for customers, helping them design experiments, troubleshoot, and achieve reliable results. We do this because that's what we'd want if we were in the lab. Because, actually, we are.

Immunohistochemistry Group, Danvers, MA USA



Cell Signaling  
TECHNOLOGY®



# Cell Signaling

TECHNOLOGY®

## UNITED STATES

Hours: M–F 8:30AM to 8:00PM [EST]

Orders: 877-616-2355 | [orders@cellsignal.com](mailto:orders@cellsignal.com)

Support: 877-678-8324 | [support@cellsignal.com](mailto:support@cellsignal.com)

[www.cellsignal.com](http://www.cellsignal.com)

## CHINA

Tel: +86-21-58356288

Support (China): 4006-473287/GreatQ | [tech@cst-c.com.cn](mailto:tech@cst-c.com.cn)

Support (Asia Pacific): [csttechasia@cst-c.com.cn](mailto:csttechasia@cst-c.com.cn)

[www.cst-c.com.cn](http://www.cst-c.com.cn)

## EUROPE, MIDDLE EAST & AFRICA

Tel: +31 (0)71 568 1060

E-mail: [eusupport@cellsignal.eu](mailto:eusupport@cellsignal.eu)

[www.cellsignal.com](http://www.cellsignal.com)

## JAPAN

Tel: 03-3295-1630

Support: [info@cstj.co.jp](mailto:info@cstj.co.jp)

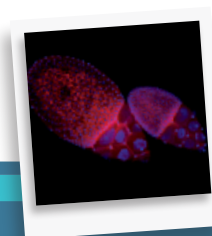
[www.cstj.co.jp](http://www.cstj.co.jp)

## AUTHORIZED DISTRIBUTORS

See a full list of our distributors online.

[www.cellsignal.com/Distributors](http://www.cellsignal.com/Distributors)

[WWW.CELLSIGNAL.COM](http://WWW.CELLSIGNAL.COM)



## FRONT COVER IMAGE:

$\beta$ -Tubulin (9F3) Rabbit mAb  
(Alexa Fluor® 555 Conjugate)

#2116: Confocal IF analysis of  
Drosophila egg chambers using #2116.